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THE

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STUDIES ON VISCERAL REGENERATION IN SEA-STARS. II. REGENERATION OF PYLORIC CAECA IN ASTERIIDAE, WITH NOTES ON THE SOURCE OF CELLS IN REGENERATING ORGANS[1]

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In a previous article (Anderson, 1962) it was shown that sea-stars from which pyloric caeca had been operatively removed were capable of making good progress towards the eventual replacement of these organs within the relatively brief period of time (8 weeks) covered by the experiments reported. These earlier studies demonstrated that in *Henricia leviuscula* (Family Echinasteridae) the preliminary steps in regeneration of the caecum produced a cylindrical, tubular outgrowth advancing between suspending mesenteries from the proximal stumps of the severed pyloric ducts. Presumably the Tiedemann's pouches, flagellary pumping organs which are normal appendages of the oral sides of the caeca, are subsequently elaborated by modification of the floor of the tubular outgrowth. Histological study of the regenerating organs showed a clear distal-proximal gradient of differentiation, with no trace of the precociously differentiated tip characteristic of the parietal parts of a regenerating ray. As no zone of cellular proliferation could be identified in the *Henricia* material, it was suggested that outgrowth of the regenerating caecum might involve the mobilization and incorporation of amoebocytes wandering in the connective tissues and fluid spaces of the body, reaching the regenerate by way of the mesenteric sheets always reconstituted in advance of the outgrowing tube.

Experiments similar to those already reported for *Henricia* have also been performed on three species (*Asterias forbesi, Pisaster ochraceus,* and *Leptasterias pusilla*) belonging to the Family Asteriidae. In these sea-stars the pyloric caeca are relatively simple, lacking Tiedemann's pouches, and the replacement of a functional organ after extirpation might be expected to proceed more rapidly and in less complicated fashion than in *Henricia*. It is my intention to present here a relatively brief, composite account of the results of my studies on these three species,

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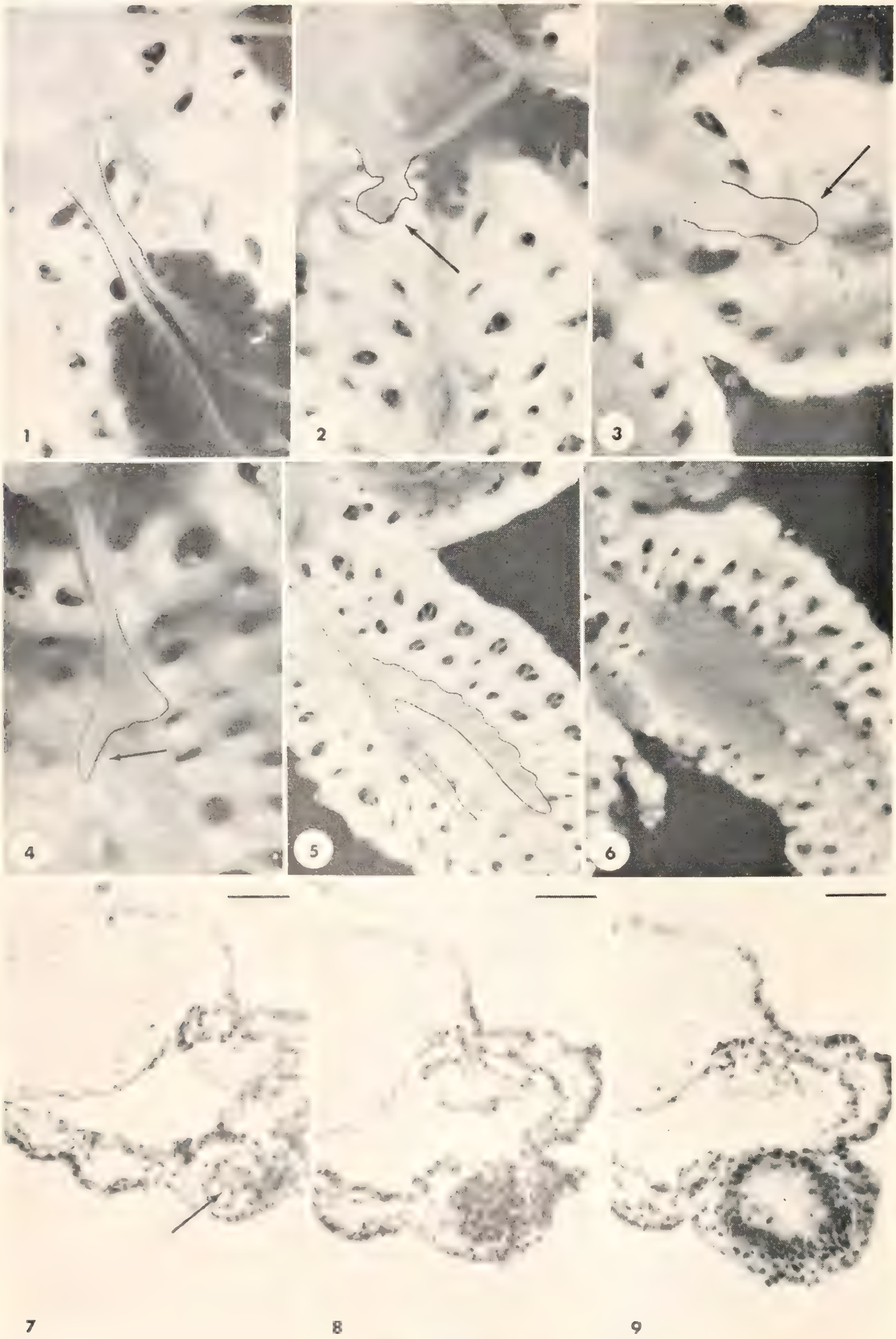


FIGURES 1-9.

with particular attention to points of comparative interest in relation to the earlier studies on *Henricia* and to points not brought out in those studies.

A further series of experiments involved the administration of tritiated thymidine to specimens of *Asterias forbesi* at a known stage in the regeneration of pyloric caeca only, or in the replacement of entire autotomized rays. Autoradiographs prepared from tissues of these animals have provided new information on the production of cells making up the regenerating parts, and the results of these experiments will be presented.

The work on the West Coast species was carried on at the Hopkins Marine Station of Stanford University, Pacific Grove, California. Once again, I record my sincere appreciation of the many kindnesses extended by the Director and staff of this station. It is a pleasure to acknowledge also the competent technical assistance of Rebecca Folsom Ferguson in experiments carried out at the Marine Biological Laboratory, Woods Hole.

Materials and Methods

Small specimens of *Leptasterias pusilla,* radius about 3 cm. or less, were collected intertidally at Mussel Point and Point Piños, in Monterey Bay; *Pisaster*

FIGURE 1. *Asterias:* single pyloric duct leading from the pyloric stomach (upper left) and branching distally to form the paired pyloric caeca, characteristic of members of the Family Asteriidae. In operative removal of the pyloric caeca this duct is severed near its origin from the pyloric stomach. (Approx. 6 ×.)

FIGURE 2. *Asterias:* stump of severed pyloric duct (arrow) one week after removal of the pyloric caeca. The diffuse dark area below marks the site of the incision in the aboral body wall and shows the extent of post-operative healing in 7 days. (Approx. 6 ×.)

FIGURE 3. *Asterias:* pyloric duct (arrow) 10 days after removal of the pyloric caeca. (Approx. 8 ×.)

FIGURE 4. *Asterias:* appearance of the regenerating duct two weeks after removal of the pyloric caeca; this shows the incipient bifurcation of the regenerate. Arrow indicates the approximate level of the cross-section shown in Figure 26. (Approx. 12 ×.)

FIGURE 5. *Asterias:* progress in regeneration three weeks after removal of the pyloric caeca. Note that one of the branches is longer and broader than the other and shows developing folds in its wall. The sections shown in Figures 7 through 11 were made from this specimen. (Approx. 3 ×.)

FIGURE 6. *Asterias:* regenerated pyloric caeca 5 weeks after removal of the original pair. Note the extensive folding and branching of the walls of both replacement organs; from all indications the regenerate is now fully functional. (Approx. 3 ×.)

Figures 7 through 11 represent cross-sections of regenerating pyloric caeca in the three-week specimen shown in Figure 5. Tissue fixed in Helly's fluid, sections stained in phosphotungstic acid hematoxylin.

FIGURE 7. *Asterias:* section just distal to the end of the shorter, smaller regenerating tube The mesenteric sheets hanging from the aboral body wall have fused to form a continuous tunnel; the summit of the tunnel at this level contains an accumulation of mesenchyme cells (arrow). (Scale = 25 μ.)

FIGURE 8. *Asterias:* similar section of the same regenerate just proximal to the last The dark mass of cells occupying the summit of the tunnel represents the closed end of the tubular outgrowth advancing between the mesenteric sheets. (Scale = 25 μ.)

FIGURE 9. *Asterias:* the same regenerate sectioned somewhat more proximally. The lumen is well developed even at this far distal level, and its lining epithelium consists of tall cells with flagella and a brush border, with nuclei crowded toward the base. (Scale = 25 μ.)

specimens of about the same size were obtained at Mussel Point and along the shore just north of Santa Cruz, in an area where small individuals are locally abundant. These animals were kept in running sea-water at the Hopkins Station and were fed occasionally on cracked snails and pieces of mussel flesh. Similarly small specimens of *Asterias forbesi* were provided by the Supply Department at Woods Hole and maintained in the circulating sea-water of the Laboratory; these animals were also fed on small snails and bivalves.

In most cases, operative technics for this series of experiments were as previously described (Anderson, 1962) for the studies on *Henricia*. Animals were immobilized by soaking in $MgCl_2$ solution and the paired pyloric caeca removed from one ray through a longitudinal incision in the aboral body wall. Removal of the caeca involved transecting the pyloric duct (which in these species, unlike *Henricia*, is single as it leaves the pyloric stomach; see Figure 1) and cutting or tearing the mesenteries attaching the caeca to the body wall. In a few instances, to determine the effects of partial extirpation of the organs, only one member of the pair was removed from a ray, or only intermediate or terminal portions of a member. In other cases, to check the animals' ability to repair more massive defects, the pyloric caeca were removed from all the rays. Returned to running sea-water, the animals usually recovered rapidly, and the operative incisions healed well without the use of medication or sutures.

To follow the course of regeneration, individuals were sacrificed and examined at approximately weekly intervals, beginning at the close of the first post-operative week. Observations were continued for 12 weeks on *Pisaster*, for 10½ weeks on *Leptasterias*, and for 6 weeks on *Asterias*; the extended duration of the experiments with the West Coast forms reflects the relatively slower rate of regeneration found to be characteristic of these species. Some individuals from which all of the pyloric caeca had been removed were followed for longer periods; in the case of one *Pisaster*, observations were continued for 16 weeks. These animals were checked periodically with respect to feeding behavior and ability to digest material that had been ingested.

Procedures for determining the progress of regeneration were as previously outlined and will not be described in detail here. As before, fixation of tissues was in Helly's fluid, chosen particularly in order to preserve cytoplasmic elements of the secretory cells in the regenerating organs. Decalcification involved soaking the tissues for about a week in 5% aqueous disodium EDTA, a chelating agent. Paraffin sections of the aboral body wall with attached regenerating viscera were cut at 7 to 10 μ and stained in Mallory's phosphotungstic acid hematoxylin.

For the experiments on *Asterias* utilizing tritiated thymidine, groups of animals were prepared by removing one pair of pyloric caeca from each by the standard technic. After these animals had regenerated for two weeks each received, in the body cavity of the operated ray or an adjacent ray, an injection of either 0.05 or 0.10 ml. of tritiated thymidine solution. The solutions were made up in sterile sea-water in concentrations calculated to provide 1.0, 10.0, or 100.0 μc./ml.; the total activity administered to each animal thus varied between 0.05 and 10.0 μc. Injected animals were kept separately in small bowls of cool sea-water for 3, 6, 12, or 24 hours. For comparison, four additional individuals were used which had been regenerating entire rays, not just excised pyloric caeca, for two weeks following induced autotomy. These received comparable injections of the same solutions and were maintained for 6 or 12 hours.

Following the indicated period of incubation the animals regenerating pyloric caeca were relaxed in $MgCl_2$ and carefully dissected and the extent of regeneration noted. The aboral body wall of the operated ray, bearing the regenerating parts, was pinned out on wax and flooded with Bouin's fluid. After a brief hardening period the tissue was trimmed and transferred to a vial of the same fixative and placed under partial vacuum in a desiccator. Bouin's fluid was chosen because, in addition to its efficacy in preserving nuclear details, its acidity brings about rapid decalcification of the body-wall ossicles and obviates the necessity of soaking the tissue for long periods in the customary EDTA solutions, with attendant loss of radioactive compounds. The partial vacuum promoted the escape of gas bubbles resulting from decalcification and minimized tissue distortion. After one to two days' fixation and decalcification the tissues were washed in 80% alcohol, dehydrated, embedded in paraffin by standard technics, and sectioned at $10\ \mu$. Animals regenerating entire rays were handled similarly except that they were not dissected before fixation.

Alternate slides from the sets bearing sections of these tissues were used in the preparation of autoradiographs. They were dipped in Kodak nuclear track emulsion (Type NTB-3), air-dried, sealed in light-tight boxes with a desiccant, and kept in a freezer for 7 days. They were then developed in D-19 and stained in dilute Harris' hematoxylin. The remaining slides were routinely stained in Mallory's phosphotungstic acid hematoxylin, for purposes of comparison and to reveal cytological details obscured by the stained emulsion overlying the sections in the autoradiograph slides.

RESULTS

For any one of the species studied here, the sequence of events in caecal regeneration, while not entirely uniform, is consistent and predictable within limits. For example, *Asterias forbesi,* under the conditions of these experiments, can replace a set of excised pyloric caeca with a reasonably complete regenerate within 5 to 6 weeks. The first postoperative week brings little grossly observable progress (Fig. 2) beyond closure and partial healing of the body-wall incision. By 10 days (Fig. 3) a blind tubular outgrowth appears at the end of the severed pyloric duct, and usually within two weeks this outgrowth has at least begun the process of bifurcation which is responsible for the pairing of the replacement organ. The two branches thus formed continue to elongate during the next couple of weeks, one usually extending somewhat farther than the other. At the same time (Fig. 5) they become broader and deeper and develop folds and incipient sacculations in their side walls. By 5 weeks (Fig. 6) this process has produced the complex branching side pockets characteristic of the complete organ; the caeca at this time are still somewhat smaller than those in adjacent intact rays, but another week or two of continued growth will undoubtedly bring them to their normal size.

Pisaster and *Leptasterias* evidently replace extirpated caeca through the same processes observed in *Asterias,* as structurally similar stages can be found in all three species. In the Californian forms, however, the rate of regeneration is considerably slower. Figure 12, for instance, shows a *Pisaster* regenerate typical [illegible] of 8-week specimens examined; comparison with Figure 5 makes it [illegible] that *Asterias* has made more progress in three weeks than *Pisaster* made [illegible]. The nearly complete pair of replacement caeca shown in Figure 14 for *Pisaster* is structurally about equivalent to the *Asterias* regenerate pictured in Figure 6; it will be noted, however, that this represents a 5-week regenerate in *Asterias* but a 12-week growth in *Pisaster*. The rate in *Leptasterias* is closer to that in *Pisaster* than to *Asterias;* the beginning of bifurcation is not seen until four weeks. The 5- and 6-week regenerates shown in Figures 16 and 17, while in some respects better developed than an 8-week *Pisaster* (Fig. 12), are much simpler than the three-week *Asterias* caeca, with their walls already somewhat folded, seen in Figure 5. The oldest *Leptasterias* regenerate in the series, the 10½-week specimen in Figure 18, does not approach the structural complexity of the 12-week *Pisaster* (Fig. 14) or the 5-week *Asterias* (Fig. 6).

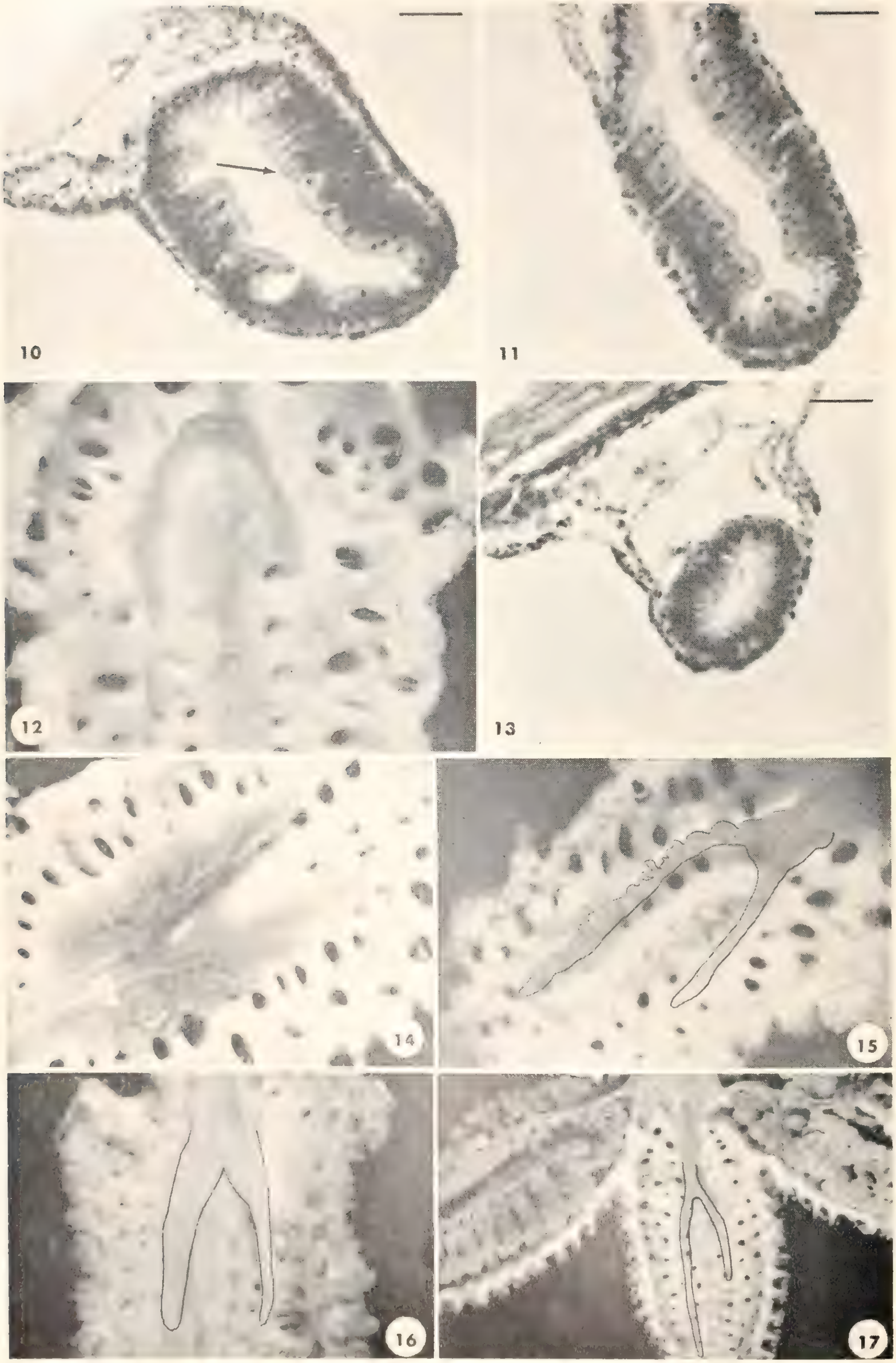
Rate differences aside, however, histological studies show that at equivalent morphological stages, irrespective of age, regenerates of the three species under investigation are very similar to one another and are obviously formed through similar processes. Therefore, in a general description of the events in regeneration and differentiation, illustrative examples drawn from any of the species should be broadly applicable to all. Further, in any regenerate, of whatever age, the youngest, simplest, most recently added part lies at the distal tip and is followed in a proximal gradient by regions successively older, more mature, and more complex. It should be borne in mind that the growing tip of an advanced regenerate is histologically equivalent to the corresponding region in any younger regenerate, and that following serial sections of a single specimen from tip to base affords an opportunity of studying, in what amounts to a time-sequence, the processes of growth and differentiation in the developing organ.

After the repair of the operative incision, the earliest internal events in caecum regeneration involve fusion of the torn edges of the mesenteries to form a continuous tunnel. This consists of double mesothelial sheets separated by a thin connective-tissue and mesenchymal layer. There is no general hypertrophy of the peritoneum, as previously observed in *Henricia* (Anderson, 1962); in *Leptasterias* the mesothelial cells immediately overlying the regenerate appear unusually tall and basophilic (Figs. 19, 20), but this is not at all comparable to the widespread involvement of the peritoneum seen in *Henricia.* Under ideal circumstances the course of the mesenteric tunnel corresponds precisely to the lines of attachment of the original caeca, and thus the tunnel forks at the point of bifurcation of the original organ and continues into the ray as a pair of parallel structures, one on either side of the healing incision. Distally these are low, flat ridges, but proximal to the bifurcation they become more elevated and join the mesenteries supporting the healed-over stump of the pyloric duct. As in *Henricia,* the preliminary formation of these tunnels provides a guide for the subsequent outgrowth of the regenerating caecum. Occasional cases of deranged regeneration can often be explained as resulting from unusually severe damage to the original mesenteries, and to the peritoneum with which they are continuous, during the operation in which the pyloric caeca were excised.

With at least the proximal portions of the guiding mesenteries reconstituted, replacement of the caecum itself begins. Sections show that the outgrowth observed in the dissections consists of a cellular tube, continuous with the lining of the pyloric duct, advancing through the mesenchymal layer between the mesothelial sheets at the summit of the tunnel. As this outgrowth reaches the bifurcation of the tunnel, it broadens, and then from its two outer corners a pair of smaller tubes appears and invades the parallel tunnels leading distally into the ray. Since the distal portions of regenerates of any age present the same histological features, the nature of these very young outgrowths can be understood by reference to sections of older ones. Immediately in advance of the growing tip there appears an accumulation of mesenchyme cells, as shown in Figure 7. The outgrowth itself comprises a solid mass of cells only at its extreme end (Figs. 8, 19); within a very short distance proximally a lumen appears, continuous with that of the pyloric duct (Figs. 9, 13, 20). Very close behind the tip the cells lining the lumen show many of the characteristics of those found in the epithelium of the mature organ; they are relatively tall, with nuclei crowded toward the basal end and with flagella and a brush border at the free end, as seen in Figure 9. Further, even very near the end of the tube, and in regenerates of only 10 days' growth, the epithelium contains secretory elements like those found in the normal caecum; zymogen cells (Fig. 13) and mucous goblets (Fig. 20) appear in the epithelium almost as far out as the lumen extends.

As the tip of the tube progresses distally, it thus establishes and leaves behind at more proximal levels the basic cell groups out of which the mature regenerate develops. Figures 10 and 11, representing sections some distance back from the tips of the two regenerating tubes seen in Figure 5, show the characteristics and relationships of the cell layers making up the forming organ. In the tall columnar epithelium lining the lumen, both zymogen and mucous gland cells are present in increased numbers as compared with more distal areas. The mesothelia covering the outer surfaces of the regenerate have become stretched and flattened as the diameters of the epithelial tubes have increased and their proportions have altered. Between the lining epithelium and the covering peritoneum, what appear to be remnants of the mesenchymal accumulations in the tunnel are spread in a thin layer, forming the probable antecedents of the subepithelial connective-tissue and muscle components of the developing organ.

Figures 21 and 22 show, respectively, sections of distal and proximal levels of the *Leptasterias* regenerate seen in Figure 16. The thin-walled, cavernous nature of the tubes at these levels is evident, but it will be noted that in Figure 22, at the point of bifurcation of the regenerate, localized thickenings have produced inward bulges in the lining, representing the beginnings of the folds and pouches characteristic of the mature organ. Figure 23, showing sections through the branches of a much older and better-developed regenerate, illustrates a further stage in the pouching process as well as a second very significant change. This involves localization of differentiated cell types in specific areas of the lining, as a result of which the side-walls, forming the precursors of the glandular pouches of the caecum, come to contain practically all of the secretory elements of the epithelium. In contrast, the roof and floor of the regenerate, which will constitute the median duct of the caecum, are lined with relatively unspecialized flagellated cells engaged in current production and responsible for maintenance of the characteristic pattern of circulation in the contents of the organ. That this circulation has already been set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FIGURES 10–17

up even in very immature distal regions of a regenerate is indicated by Figure 13, which shows that debris characteristic of the external environment has been carried to the extreme end of the lumen of this regenerating tube.

There can be little doubt that the functional capacities of the regenerate are established early and progressively develop towards normalcy as the size and structural complexity of the organ continue to increase, as secretory elements differentiate in increasing abundance and become localized in the glandular pockets, as the normal patterns of movement and circulation of the fluid contents assert themselves, and as the subepithelial muscular, connective-tissue, and nervous elements develop in the wall of the caecum. It is evident that from relatively early stages the regenerate functions as an organ, even though in terms of structural and functional differentiation its immature distal extremities lag far behind the older proximal portions.

For comparison with the general process of regeneration which can now be taken as established, a smaller series of experiments involving either partial or total caecal extirpation provides instructive material. Figure 24, for example, illustrates the result of an experiment in which a single member of the pair of caeca in one ray was excised, 6 weeks previously, in *Leptasterias*. Comparison with Figure 17 makes it apparent that the presence of the intact caecum in the ray has had no demonstrable effect on the rate of replacement of the excised member. The caecal tube has clearly grown out from the point of amputation of the original, following the path established by the mesenteric attachments of the missing portion. It is probably only coincidental that in the 6-week period provided, the regenerate has extended to a point almost exactly level with the tip of the normal member. Histologically, the tubular organ at this stage is in all respects comparable with

FIGURE 10. *Asterias:* the three-week regenerate at a still more proximal level. The tube here is taller than broad, and in addition to the conspicuous mucous goblet, the epithelium contains numerous zymogen cells lying in the region indicated by the arrow. Note also the large nuclei scattered high in the epithelium. (Scale = 25 μ.)

FIGURE 11. *Asterias:* cross-section of the larger of the two regenerating tubes shown in Figure 5. The epithelium contains numbers of zymogen cells and mucous goblets; except for the presence of the unusually numerous large nuclei in the clear areas bordering on the lumen, it appears very similar to the normal caecal epithelium. (Scale = 25 μ.)

FIGURE 12. *Pisaster:* specimen dissected after 8 weeks' regeneration following removal of pyloric caeca. The forked regenerate extends distally from the single pyloric duct at top center. Each fork is a deep tube with walls only weakly folded. (Approx. 6 ×.)

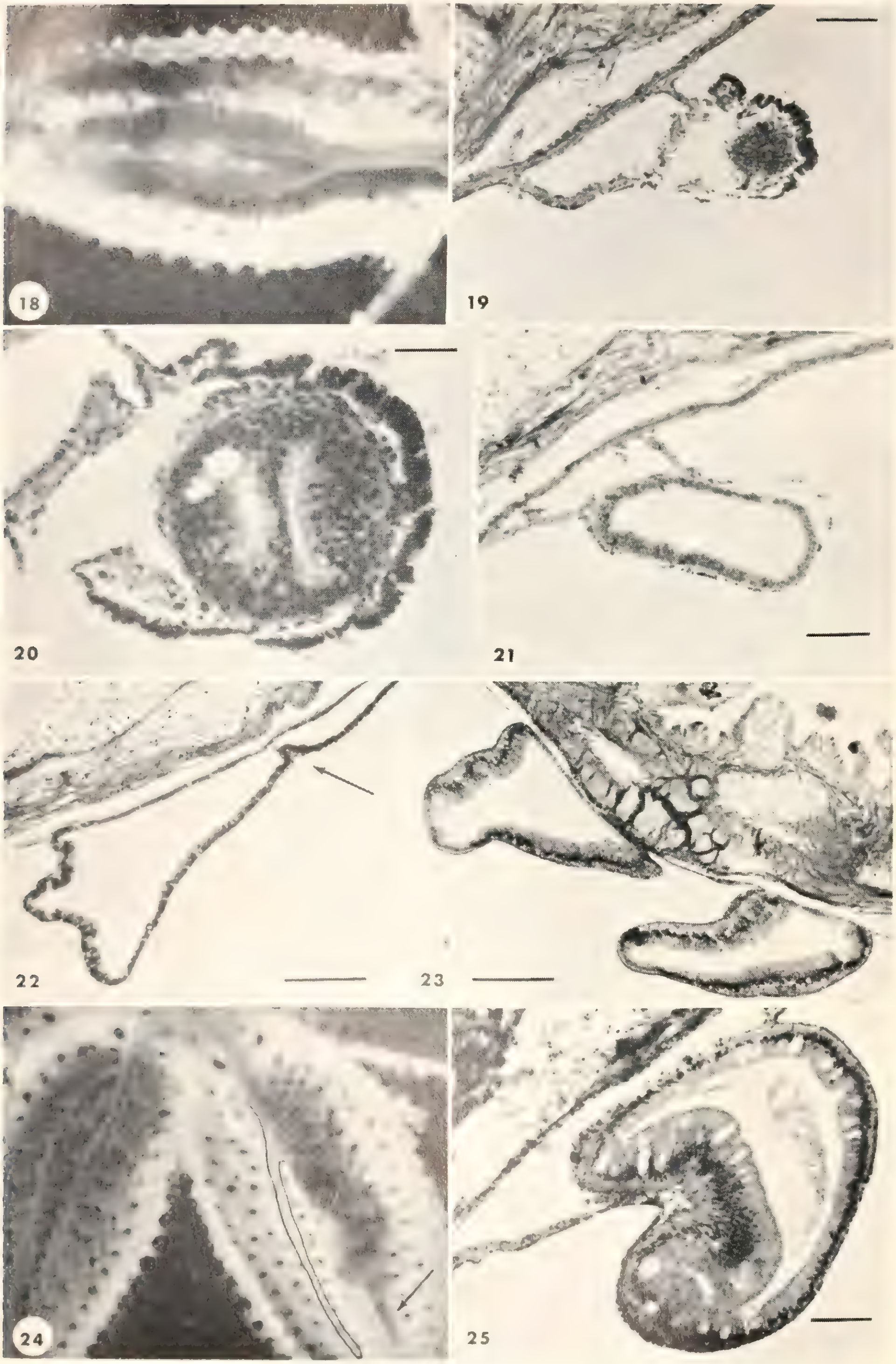
FIGURE 13. *Pisaster:* cross-section of an 11-week regenerate, far distally. As in *Asterias* (*cf.* Fig. 9), the regenerate advances between mesenteric sheets in a guiding tunnel. The lining epithelium shows differentiated secretory cells, as well as flagella and brush border, and the lumen contains detritus from the external environment. PTA hematoxylin. (Scale = 25 μ.)

FIGURE 14. *Pisaster:* well-developed regenerate 12 weeks after removal of the original pair of caeca. Structurally, this is perhaps slightly more advanced than the 5-week *Asterias* regenerate shown in Figure 6. (Approx. 4 ×.)

FIGURE 15. *Pisaster:* one pair of regenerating caeca in an animal from which all 5 pairs had been removed almost 12 weeks previously. One member of this pair shows developing folds and wrinkles, but comparison with Figure 14 shows that the replacement of 5 pairs of caeca is slower than the regeneration of a single pair in the same species. (Approx. 5 ×.)

FIGURE 16. *Leptasterias:* dissection of a 5-week regenerate. The sections shown in Figures 19 through 22 were made from this specimen. (Approx. 8 ×.)

FIGURE 17. *Leptasterias:* progress in regeneration 6 weeks after removal of the pyloric caeca. As in most cases, one branch extends beyond the other. The size and relationships of the intact pyloric caeca can be seen in the adjacent unoperated rays. (Approx. 4 ×.)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FIGURES 18-25.

either of the paired regenerates of the same age shown in Figure 17. Conversely, the presence of the regenerating caecum has exerted no demonstrable effect on the normal member of the pair. Although in Figure 24 the bulk of this organ appears somewhat less than that of a normal caecum in the adjacent ray, this probably results from a difference in the way in which the organ lies in the opened coelomic cavity; approximately the same number of glandular pouches is present in all. Obviously, removal of one member of the pair has provided the remaining member additional space in which to lie. As Figure 25 shows, the extreme distal portion of the intact organ presents normal histological characteristics and shows no indications of stimulated growth or of de-differentiation, as might perhaps have been expected.

A different sort of experiment, not illustrated in the figures, provides additional information bearing on questions of growth-gradients in the regenerating system. In *Leptasterias,* one ray was opened and the proximal portion of one member of its pair of caeca was excised, leaving intact a 1-cm. length of its distal end. At the same time, the proximal and distal portions of the other member were removed, leaving the middle part in place. Opened again after a 5-week period, the animal showed significant changes. Both of the isolated portions of the two caeca, which had been left attached only by their respective mesenteries, had undergone considerable reduction and resorption, although remaining identifiable and histologically recognizable. Neither had produced regenerative outgrowths in either direction. But while these isolated distal segments had not themselves contributed any new growth, neither had they exerted any inhibitory effects on normal events in regeneration; the usual mesenteric tunnels had formed proximally

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FIGURE 18. *Leptasterias:* well-developed regenerate, with simple sacculate foldings in its walls, 10½ weeks post-operative. The section shown in Figure 23 was made from this specimen. (Approx. 6 ×.)

FIGURE 19. *Leptasterias:* cross-section at the extreme end of the smaller branch of the five-week regenerate shown in Figure 16. The crowded cells occupying the summit of the tunnel mark the closed end of the advancing tube. This section is comparable in all respects with that shown in Figure 8, for *Asterias,* with the exception of the characteristic hypertrophy of the peritoneum over the regenerate in *Leptasterias.* Helly-fixed; PTA hematoxylin. (Scale = 50 μ.)

FIGURE 20. *Leptasterias:* section just proximal to the last. The lumen extends almost to the distal end of the regenerate, and even at this level the epithelium is well differentiated, containing conspicuous mucous goblets. PTA hematoxylin. (Scale = 25 μ.)

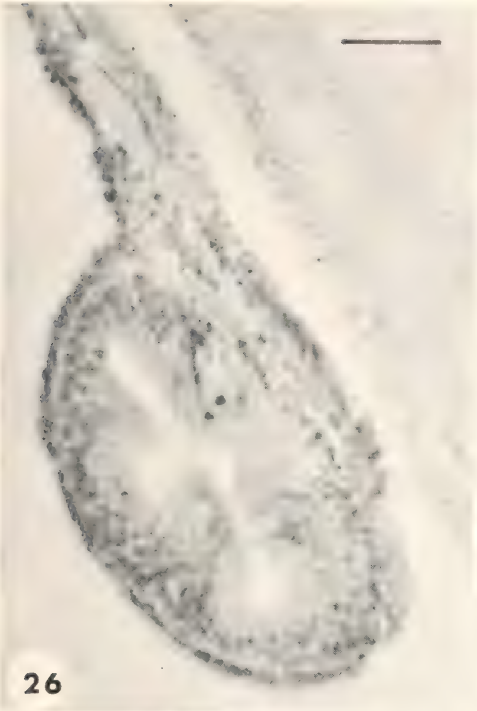
FIGURE 21. *Leptasterias:* section of the same regenerate about midway to its base. The wall is thin and consists almost entirely of the epithelium lining the tube, with abundant secretory cells. PTA hematoxylin. (Scale = 50 μ.)

FIGURE 22. *Leptasterias:* the same 5-week regenerate sectioned at its point of bifurcation. The arrow indicates the region in which the floor of the broad proximal duct rises to meet its roof and separate the two distal branches. Note thickenings of the lateral walls of the wider tube, shown here, marking incipient folds. PTA hematoxylin. (Scale = 100 μ.)

FIGURE 23. *Leptasterias:* cross-section of the 10½-week regenerate shown in Figure 18, at about midlength. Note the vertical depth of the tubes, and the histological differentiation between the side walls and the oral gutter. PTA hematoxylin. (Scale = 100 μ.)

FIGURE 24. *Leptasterias:* dissection of a specimen 6 weeks after removal of a single member of the pair of pyloric caeca in one ray. Compare the growth of this single regenerate with that shown in Figure 17, where both branches have been regenerating for 6 weeks. The arrow indicates the level of the section shown in Figure 25. (Approx. 6 ×.)

FIGURE 25. *Leptasterias:* cross-section of the distal end of the intact caecum shown in Figure 24 (arrow). The histology of this organ appears completely normal. PTA hematoxylin. (Scale = 50 μ.)



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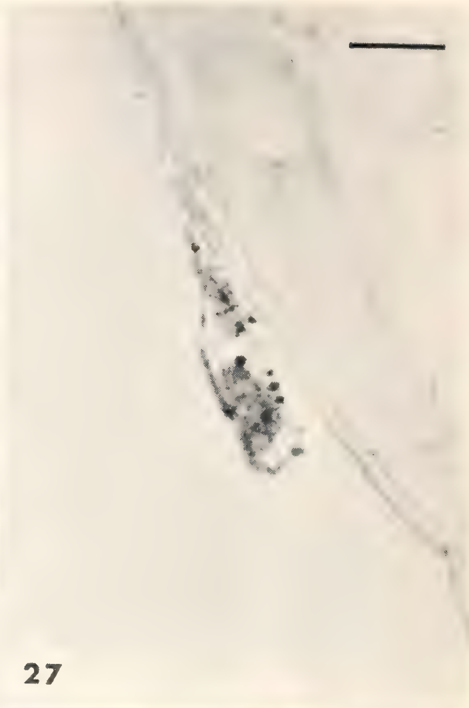

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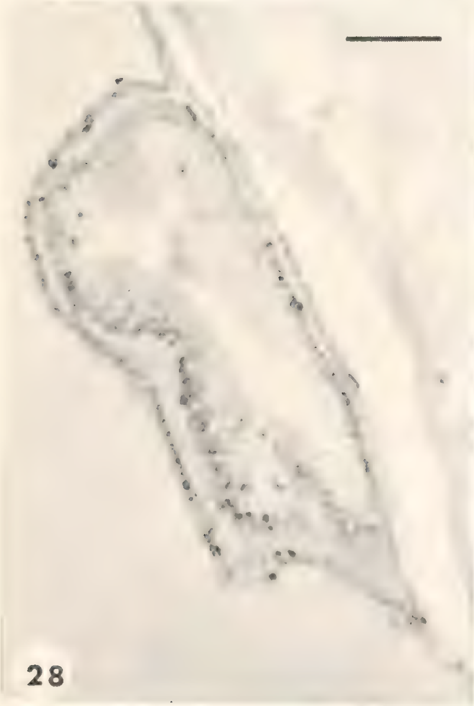

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Figures 26–29.

and extended to join with those of the distal fragments, and tubular caecal regenerates had proceeded to grow from the pyloric duct, reaching a stage comparable with the 5-week organ shown in Figure 16 (but not attaining the level of the resorbing fragment).

Other experiments, involving simultaneous removal of all 5 pairs of pyloric caeca from specimens of *Pisaster* and *Asterias*, demonstrate that these animals rapidly recover from the effects of such radical surgery on the digestive tract and are capable of making good progress, within the time limits of the experiments, towards eventual replacement of all the missing organs. The events in regeneration of individual caeca after multiple excision are the same as those described for replacement of single pairs, but the rate of regeneration is considerably slower. Figure 15 shows one of the 5 pairs of regenerating caeca in a *Pisaster* specimen just short of 12 weeks following removal of all its caeca; regeneration in the remaining rays had proceeded to an equivalent condition. Regenerative progress here obviously is slower than that shown in the 12-week specimen illustrated in Figure 14 and appears about comparable with that of the 8-week single-pair regenerate shown in Figure 12. The rate of replacement in similarly operated specimens of *Asterias* is also reduced although characteristically more rapid than in *Pisaster*, as previously noted.

Sea-stars deprived of their pyloric caeca are incapable of digesting food until the caeca are replaced. This fact makes it possible to chart the return of function after total caecal extirpation, by observing the performance of specimens in repeated feeding trials. The following observations describe the feeding behavior of an operated *Pisaster* at the indicated post-operative intervals:

11 days: offered food, ignores it
18 days: offered food, ignores it
19 days: attacks small mussel, later releases it unopened
3 weeks: attacks and opens small mussel, remains humped over it several hours but produces no evidence of digestion
4 weeks: repeats 3-week behavior, with same result
7 weeks: opens small mussel, partially digests soft parts during several hours of feeding
11 weeks: opens mussel, completely digests soft parts

FIGURE 26. *Asterias*, autoradiograph: cross-section of a two-week regenerate (see arrow in Figure 4) fixed three hours after injection of 0.5 μc. of thymidine-H^3, sectioned and prepared as described in the text. This is a normally growing regenerate, but it is evident that nuclear labeling is minimal or practically non-existent. Bouin, Harris hematoxylin. (Scale = 25 μ.)

FIGURE 27. *Asterias*, autoradiograph: two-week regenerate, section showing mesenteric tunnel distal to advancing tube. The specimen was fixed 6 hours after injection of 1.0 μc. of thymidine-H^3. Note the localization of labeled nuclei in the mesenchyme and peritoneum involved in tunnel formation. Bouin, Harris hematoxylin. (Scale = 50 μ.)

FIGURE 28. *Asterias*, autoradiograph: two-week regenerate, cross-section in proximal region of advancing tube; specimen fixed 12 hours after injection of 1.0 μc. of thymidine-H^3. Labeled nuclei appear in all cell layers of the regenerate but are particularly abundant in the lining epithelium where folds are developing. Bouin, Harris hematoxylin. (Scale = 50 μ.)

FIGURE 29. *Asterias:* oral aspect of a replacement ray known to have been formed within two weeks, following induced autotomy of the original. Approximately 15 pairs of tube feet have been produced, in addition to the terminal tentacle. This is the stage of regeneration represented in subsequent figures. (Approx. 10 ×.)

When this specimen was dissected within a few days of the last recorded observation, it proved to contain forked, tubular, somewhat folded regenerates in all rays, similar to those found in the specimen shown in Figure 15. In the 5 similar experiments performed, *Pisaster* specimens showed consistently that digestive functions were effectively re-established within 11 to 12 weeks. For comparison, the same experiments on *Asterias* indicated return of digestive function sometime between the fourth and sixth post-operative week. It may be noted as of passing interest that after a variable period of recovery all specimens acted "hungry" in the presence of food, and exhibited normal feeding behavior, for some time prior to the return of their ability actually to digest food enveloped by, or brought into, the cardiac stomach.

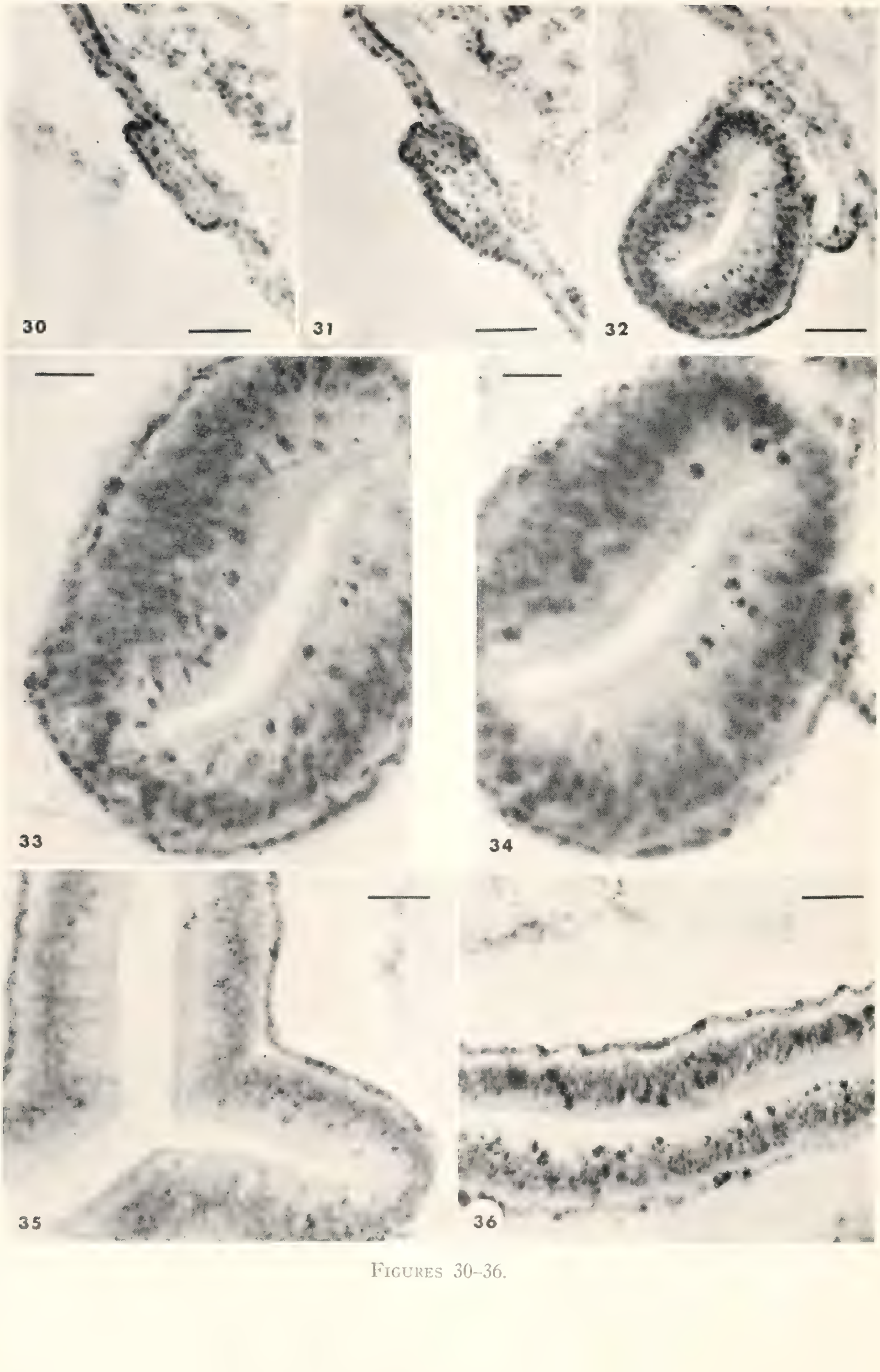
Turning now to the thymidine-H^3 experiments with *Asterias,* we may first note the technical point that 0.5 μc. total activity, available to the tissues for three hours, produces practically no nuclear labeling in the organism (Fig. 26). Doubling the total activity administered, and increasing the incubation time to 6 or 12 hours, brings about the abundant labeling shown in Figures 27 and 28. These figures illustrate clearly the very significant fact that thymidine incorporation, marking sites of impending or recent cell division, occurs at all levels of the two-week regenerate, from the elements of the far distal tunnel (Fig. 27) to the proximal outgrowth of the pyloric duct (Fig. 28). As these figures show, background activity is very low, and labeling of nuclei appears almost entirely limited to tissues involved in the regeneration of the caecum. The results of the entire series of experiments are consistent and demonstrate that mitotic proliferation proceeds very actively in the growing regenerate.

These observations are confirmed by the results of parallel experiments involving regeneration of pyloric caeca within regenerating rays. Figure 29 is an oral view of a two-week regenerate replacing an autotomized ray. The gradient of growth and differentiation in this young ray is clearly shown. Structures of the distal tip, while not yet of full size, are well differentiated; the most proximal features, such as tube feet, spines, and ossicles, are largest and oldest, and a gradient can be followed from the base of the new ray to a region just behind its tip, which contains the youngest structures and the zone of growth where they are formed. Sections of such a regenerating ray show that without question its visceral components are replaced through a sequence of events precisely comparable to those observed in the regeneration of excised pyloric caeca. Far distally, one encounters a low ridge-like structure (Fig. 30) which is continuous proximally with a typical mesenteric tunnel (Fig. 31). More proximally still, the summit of this tunnel is occupied by a tubular caecal outgrowth from the pyloric duct, and sections of such an outgrowth (Fig. 32) show that it is quite comparable to similar levels of previously observed replacement organs (Figs. 9, 13, for example). Further, as demonstrated in Figures 30, 31, and 32, the components of regenerating pyloric caeca within regenerating rays show the same autoradiographic evidence for mitotic activity as that found in material from the excised-caecum experiments.

But sections of regenerating caeca prepared for autoradiographs present not only the indirect evidence of thymidine uptake indicative of mitotic activity, but also conspicuous and unmistakable mitotic figures themselves. It will be recalled that this material was fixed in Bouin's fluid, while in earlier experiments all fixation was in Helly's fluid. Restudy of sections of the Helly-fixed tissues reveals structures in the regenerating epithelium that can be interpreted as poorly-fixed mitotic nuclei; some of these may be identified in Figures 9, 11, 13, and 20, for example. In both the excised-caecum and whole-ray regenerates, particularly in *Asterias,* the advancing epithelial tube of the developing organ displays numbers of large, clear, often somewhat granular nuclei. These are especially numerous in the extreme distal regions of the tube, where most of the nuclei present appear to be of this type; proximal to the growing tip, in areas marked by differentiation of the epithelium into its specialized components, the cells with large nuclei gradually decrease in abundance, although remaining numerous and conspicuous. Here they lie at all levels in the epithelium, interspersed among the typical small, dense, basally crowded nuclei of the columnar cells lining the lumen; they are especially noticeable in the clear cytoplasmic zone above the nuclear band. It is apparently with the large nuclei that thymidine uptake in the epithelium is most frequently associated, particularly in the distal region of the regenerate, and the localization of unmistakable mitotic nuclei, as shown in Figures 32, 33, and 34, indicates that it is these cells, high in the epithelium, that are dividing most actively. It is evident that the majority of mitotic figures here are oriented parallel with the surface of the epithelium, and as a consequence new cells will be added in such a way as to contribute to the increasing diameter of the caecal tube behind the growing point.

Radioactivity in the epithelium is not altogether limited to the vesicular nuclei but is often found also in association with the small, dense nuclei of the differentiated cells. This is particularly evident in the older regions of the regenerate; note, for instance, the distribution of grains among the basal nuclei in the proximal section shown in Figure 35. There appears to be no zone in the epithelial lining of the two-week excised-caecum regenerate where mitotic activity can be said to occur with maximum intensity; the concentration of heavily labeled nuclei and of mitotic figures is greater a millimeter or two behind the solid tip of the outgrowth than in the tip itself, but it is noteworthy that cell division continues throughout the length of the tube, occurring even in the older, more proximal levels where differentiation has produced an essentially mature epithelium. As seen in Figures 28 and 35, evidence of mitotic activity is concentrated in the regions of the expanding caecum involved in the production of outfolding glandular pockets. The replacement caecum within a two-week whole-ray regenerate has not reached the stage of development characterized by wall folding, and as Figure 36 shows, mitotic activity in this tube seems fairly uniformly distributed in both older and younger regions of the lining.

Although directly observable mitotic figures are most conspicuous in the lining epithelium, cellular proliferation in the regenerate is by no means confined to this layer. As most clearly shown in Figures 35 and 36, thymidine uptake has occurred very actively in the peritoneal layer covering the outgrowing tube, and labeled nuclei are found also in the mesothelial constituents of the mesenteries, tunnels, and distal ridges. Here, as in the lining layer of the caecal tube, activity is most abundantly associated with large, clear nuclei and is less frequently found in the smaller, more irregularly shaped nuclei of typical cells of the peritoneum. The occurrence of labeling in the mesenchyme is less obvious; some activity is apparent among the mesenchyme cells forming the core of the far distal ridge in Figure 30, and perhaps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Figures 30–36.

in the tunnel shown in Figure 31. The resolution of the radiographs being what it is, however, it is impossible to state with confidence whether the developed grains scattered in the region between the lining epithelium and the covering peritoneum, at more proximal levels, actually pertain to nuclei of the mesenchymal cells, which are spread very thinly here. As the figures indicate, these grains almost always appear near heavily labeled nuclei of the peritoneum.

Discussion

No significant differences appear when events in caecal regeneration are compared among the three asteriids studied. The rate differences encountered in the different experimental series are almost certainly expressions of temperature effects. According to station records (D. P. Abbott, personal communication), mean monthly sea-water temperatures at the Hopkins Marine Station for the period occupied by the regeneration experiments on *Leptasterias* and *Pisaster* ranged between 14 and 16° C. In contrast, mean surface water temperatures at Woods Hole during the months covering the *Asterias* experiments averaged about 20° C. (D. Bumpus, WHOI sheets). It is thus not surprising that regeneration in *Asterias* should be found to proceed so much more rapidly than in the other asteriids studied. The rate in *Henricia* (Anderson, 1962) is comparable with that in the other West Coast forms, producing nothing more advanced than simple tubular regenerates in the 8-week period covered by the experiments. The peculiar involvement of the peritoneum in *Henricia,* lacking in asteriids, has already been mentioned. Beyond this, however, one may conclude that the asteriid experiments, carried to the point at which essentially complete, normal, and functional organs have been produced, reveal the kinds of changes that could be expected if the brief series of experiments on *Henricia* were extended to cover the later stages of regeneration. One would expect, of course, that some particular development in

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These figures are autoradiographs of sections showing regeneration of pyloric caeca within regenerating rays like that in Figure 29. Specimens were fixed 12 hours after injection of 1.0 μc. of thymidine-H^3 and prepared as described in the text.

FIGURE 30. *Asterias:* cross-section, far distally in the ray, showing localization of labeled nuclei in mesenchyme and peritoneum of a ridge forming in advance of the mesenteric tunnel. Bouin, Harris hematoxylin. (Scale = 25 μ.)

FIGURE 31. *Asterias:* same specimen, more proximally. The mesenteric tunnel has formed at this level and shows abundant labeled nuclei and mitotic figures, particularly in the peritoneum. Bouin, Harris hematoxylin. (Scale = 25 μ.)

FIGURE 32. *Asterias:* same specimen, more proximally still; cross-section of tubular regenerate. Labeled nuclei and mitotic figures are apparent in the peritoneum as well as in the lining epithelium. Compare Figures 9, 13. Bouin, Harris hematoxylin. (Scale = 25 μ.)

FIGURES 33 AND 34. *Asterias:* same specimen, sections similar to the last, in detail. The epithelium lining the regenerate is well differentiated; large nuclei high in the epithelium are mitotically active; labeled nuclei are numerous also in the peritoneum. Bouin, Harris hematoxylin. (Scale = 10 μ in both figures.)

FIGURE 35. *Asterias:* same specimen, sectioned more proximally where the tubular regenerate has developed folded walls. Note that labeled nuclei are scattered in all layers, apparently including the subepithelial mesenchyme. Bouin, Harris hematoxylin. (Scale = 25 μ.)

FIGURE 36. *Asterias:* longitudinal section of the tubular regenerate in a similar specimen to the last, similarly prepared. Heavily labeled nuclei in all layers, relatively uniformly distributed through the length of the tube, indicate that cellular proliferation is not limited to the advancing tip. Bouin, Harris hematoxylin. (Scale = 25 μ.)

the floor of the regenerate would eventually lead to the formation of Tiedemann's pouch, with its specifically oriented flagellary channels—a structure not produced in the other forms. Such changes could be interpreted as simply an extension of the process of cell localization observed in all the other species, as a result of which the secretory cells become segregated in the incipient glandular pouches and the more generalized current-producing cells come to be concentrated in the roof and floor of the median duct. In *Henricia* the final disposition of glandular cells is somewhat different (Anderson, 1960).

Studying the regenerative process in all the species observed, whether the new pyloric caecum is replacing an operatively-removed organ or is growing in connection with the regeneration of an autotomized ray, one can only be struck by the aptness of Hyman's simple statement (1955, p. 314): "The pyloric caeca are replaced by outgrowth from the old ones. . . ." In all cases, after a period of reorganization, whatever remains as a stump of the original organ produces an outgrowth that always appears as a simple epithelial tube, continuous with its own lining layer, which secondarily bifurcates and forms pockets. Hyman's statement continues to the effect that regeneration occurs in the same manner as post-larval growth; with this in mind, some descriptions of the events involved in the original formation of the caeca will be of interest, for purposes of comparison. Cuénot (1887), for example, refers briefly (p. 33) to the development of the pyloric caeca, pointing out that morphologically they are extensions of the gastric sac (*sac stomacal*); he gives a drawing of a developing caecum from a young *Astropecten* (Plate III, fig. 5), describing this as a tubular projection from the gastric sac without definite folds but with the same histology as that of its parent organ. Gemmill (1912), describing the development of *Solaster endeca*, gives a full account of the origins and relationships of the pyloric caeca (p. 40): "The paired radial diverticula appear as folds on the roof of the stomach which converge towards a central point. . . . The outer extremities of the folds . . . become elevated from the surface and grow out as blind pouches into the rays. . . . During the fourth month the radial diverticula begin to broaden at their outer ends. This is followed by bifurcation at these ends, with accompanying division of their suspensory mesenteries and of the pockets of epigastric coelom contained therein." Other accounts, notably those of MacBride (1896, 1914), provide generally comparable descriptions.

The similarity between embryonic or post-larval development, as outlined in the foregoing passages, and the events involved in regeneration of the organs is clearly evident in the account by Yamazi (1950) of regeneration after autotomy in the fissiparous, multirayed sea-star *Coscinasterias acutispina*. In this species the normal number of rays is 8; full-grown individuals divide across the disk, and each daughter individual then grows four small replacement rays. The pyloric stomach repairs itself, gives off a rectal sac, grows to the edge of the disk, and produces a small, tubular outgrowth leading into each of the four new rays. As Yamazi describes further changes (p. 182): "The pyloric caecum soon furcates into two hollow canals at the position of the mesenteries placed on the median line of the ray cavity. The stalk of these branches remains as a common duct. . . . The branches which are originally simple canals, issue laterally into two series of short lobular branchlets. Meanwhile, the median canal becomes flattened laterally."

The events thus described, and the structural features and changes illustrated in Yamazi's drawings, are obviously very closely similar to post-larval development as described by others; they also resemble very markedly the kinds of regenerative changes found in the present studies of asteriids.

It will be noted that Yamazi's statement refers briefly to a relationship between caecal outgrowths and the positions of the mesenteries in regeneration, and Gemmill's description of post-larval growth, quoted above, indicates that such a relationship characterizes the original formation of the organs also. On this subject Gemmill has more to say (p. 34): "As the paired diverticula of the enteron grow out, pockets from the epigastric coelom extend outwards along their aboral aspect, and these pockets bifurcate as the diverticula themselves become divided into two . . . the paired radial extensions of the mesentery . . . do not become broken up into fibers, but remain as the boundary-walls of the epigastric coelomic pockets, which in the adult lie above the paired radial diverticula of the stomach." As a result of the present studies, and those reported earlier on *Henricia,* we are now in a position to understand that in caecal regeneration the paired mesenteries do not simply *accompany* the outgrowing regenerate. Rather, the positioning and bifurcation of the mesenteric tunnels (and of the pockets of epigastric coelom which they enclose) are events that occur in advance of the outgrowth of the caecal tube in each case. In regeneration of excised caeca, it is easy to visualize how the mesenteric tunnels form through the zippering-up, so to speak, of the remnants of the pre-existent paired mesenteries. What is perhaps more intriguing is the fact that in regeneration of an entire ray the mesenteric tunnels must form anew, traversing the newly formed aboral body wall, as in the original growth of the ray. The future mesenteric tunnel is represented far distally in the new ray by the low ridge which will later become elevated and contain a coelomic extension. Arising in this way, the tunnel must bifurcate at the appropriate level so as to guide the caecal tube in its outgrowth, and in its subsequent broadening and bifurcation. What it is that guides the formation of the tunnel is not apparent.

The partial-extirpation experiments on *Leptasterias* contribute to a demonstration that caecal outgrowth, even with completely re-formed guiding mesenteries in place, occurs only in an outward direction, and only from centrally connected remnants or stumps of excised organs. They show also that remnants of an intact caecum, so long as they are centrally attached, are not noticeably utilized as a source of materials for the replacement of missing portions. Distally isolated fragments are resorbed without exerting any demonstrable effect, inhibitory or otherwise, on the progress of regeneration from the central stump. The limited series of experiments does not, however, provide an illustration of what interaction might occur when a centrally attached caecal outgrowth, advancing in its guiding mesentery, encounters a resorbing, distally isolated fragment.

The ability of *Pisaster* and *Asterias* (and presumably other species as well) to replace all pyloric caeca simultaneously after total extirpation is noteworthy. Excision of all the caeca removes the only recognized source of digestive enzymes and thus precludes the possibility of effective feeding until functional organs can be regenerated. The observations on post-operative feeding behavior of caecumless specimens confirm the supposition (previously advanced for *Patiria;* see Anderson, 1959) that the caeca are indispensable for the digestive process. At the same time, removal of the caeca eliminates the major storage organs for nutritional reserves, against which the animal would normally draw when for any reason feeding is not taking place. The energy sources for maintenance of metabolism during the period of enforced starvation pending caecum regeneration have not been identified. One can only assume that, in view of the invariably successful and relatively rapid replacement of the excised organs, subsidiary stores in the body wall and elsewhere must provide adequate reserves.

As previously noted (Anderson, 1962), growth patterns in regenerating pyloric caeca are different from those in the surrounding body wall. Yamazi's work (1950) on regeneration in *Coscinasterias* provides a graphic illustration (see his Figure 4, page 183) of the precociously developed distal structures in the body wall, advancing ahead of the zone of growth which establishes the age-gradient between itself and the base of the ray. Yamazi shows, and the present study of asteriids confirms with additional details, that there is no comparable differentiated region at the tip of the outgrowing caecum. Histologically, the tip of the caecum is the simplest and youngest region; it is noteworthy, however, that differentiation of the lining epithelium begins almost immediately behind the advancing tip, with the production of secretory cells and the introduction of specialized characteristics of the epithelial cells.

In view of the conclusive evidence, both direct and indirect, revealing the intensity of mitotic activity in the regenerating pyloric caecum of *Asterias*, it is no longer necessary or valid to invoke mobilization of amoebocytes as a primary source of new cells for the replacement organ (*cf.* Anderson, 1962). Failure to recognize mitotic figures in the regenerating caecum of *Henricia* can be attributed chiefly to a technical deficiency, involving consistent use of a fixative (Helly's) chosen as ideal for cytoplasmic features but providing poor preservation of nuclear details. Also relevant is the fact that regeneration in *Henricia* is relatively slow, with mitotic activity much less prevalent than in the rapidly growing regenerates in *Asterias*. There can be little question, however, that many scattered structures in sections of *Henricia* material, originally interpreted as pycnotic nuclei, actually represent collapsed mitotic figures. Having demonstrated the widespread occurrence of cellular proliferation in *Asterias*, one has no reason to doubt that regeneration in *Henricia*, so closely similar in all other respects, must involve the same source of cells for incorporation into the developing organ.

With a heretofore puzzling question disposed of, visceral regeneration in the asteroids studied can now be compared more meaningfully with the only other case of gut-replacement in echinoderms that has been similarly investigated—that of regeneration following evisceration in holothuroids. Dawbin (1949) has shown that in *Stichopus*, beginning about 40 days after loss of the gut, mitotic activity becomes widespread in the layer of cells lining the newly developed lumen of the regenerating alimentary tract. These cells, it will be noted, form a solid cord of mesenchyme at the thickened mesentery edge, and it is in this cord that an irregular lumen gradually forms. The cells dispose themselves so as to constitute a lining for the tube, and through continuing division provide for the growth of the gut until it eventually reaches normal size. As Dawbin points out, in order to accommodate the ever-increasing diameter of the tube the covering peritoneum must, and does, add cells also; but most of the mitotic activity observed is in the cells of the gut lining. Interestingly, cell division in the regenerating caecum of *Asterias* is observed in these same two layers, and its occurrence in the intervening thin layer of mesenchyme is questionable at best on present evidence. One point of contrast deserves emphasis: in asteroids, the cell layer lining the lumen of the regenerating caecum is always continuous with the epithelium of the pyloric duct and, as we have seen, extends outward by proliferation of cells belonging to this layer. In *Stichopus,* the lining proliferates and differentiates in place, from mesenchymal accumulations, without reference to or connection with the corresponding layer of the esophagus remnant lying anteriorly (= orally) in the supporting mesentery. In this respect, of course (see discussion in Anderson, 1962), gut regeneration in *Stichopus* differs even from that in other sea-cucumbers studied (*Holothuria* and *Thyone*), where the continuity of layers is similar to that found in asteroids. The existence of these differences makes it appear that a re-study of events in visceral regeneration in holothuroids, with particular attention to histological details, localization of mitotic activity and cellular differentiation, and related matters, would provide a valuable contribution for comparative purposes.

The thymidine-H^3 experiments on *Asterias* were originally designed, in the absence of recognizable mitotic figures in material that had been studied, simply to provide evidence for the occurrence or non-occurrence of cell division in the regenerating caecum. They were not precise enough or extensive enough to serve as a basis for detailed analysis of cell-population dynamics in this system, and such an analysis has not been attempted. However, with the original objective now attained, with the added bonus of confirmation from directly observed mitotic activity in the same sections, we may note briefly some additional information provided by study of the autoradiographic preparations. The appearance of the radioactive label in such profusion throughout the tissues of the regenerating caecum, after administration simply by injection into the coelomic fluid, provides a further indication of the activity of metabolic exchange between the circulating fluid and the pyloric caeca, as already demonstrated for various nutrients by Ferguson (1964a, 1964b).

Considering the difference in general metabolic levels, the duration of the mitotic cycle in *Asterias* is undoubtedly much greater than the 40 or so hours given by Lajtha (1957) for human bone-marrow cells in culture, or the approximately 20 hours suggested as a reasonable average by Quastler and Sherman (1959) for proliferating cells in the duodenal lining of the mouse. Thus, given the 6- to 12-hour incubation time in the present experiments, it is highly unlikely that any cell in the system can have proceeded through more than one mitotic cycle in the presence of the radioactive label. Heavily-labeled nuclei must have passed through a significant fraction of their premitotic synthetic phase during the period of incubation; more lightly labeled nuclei must represent cells exposed hardly at all to thymidine-H^3, or immediately post-mitotic daughter cells carrying only a part of the label taken up by the parent nucleus. In some cases, particularly those involving 12-hour incubation periods, grain-density is so extreme over many nuclei that the mitotic status of the cells cannot be determined (*cf.* Fig. 36). Where labeled nuclei can be identified as premitotic, it has already been pointed out that they are most frequently of the large, vesicular variety with which mitotic activity seems usually to be associated, in both lining epithelium and peritoneum. These probably represent undifferentiated cells; their progeny, it would appear, either remain undifferentiated and furnish the stock for continuing divisions; or else, in the lining layer, elongate, establish contact with the basement membrane, and transform into the columnar cells typical of the developing epithelium. The fact that some activity is associated with small, dense nuclei characteristic of these differentiated cells may identify them as post-mitotic types retaining label incorporated by a parent nucleus; alternatively, it may indicate that even differentiated cells can synthesize DNA and continue to proliferate. Leblond and Messier (1958) have reported encountering copious labeling of the nuclei of goblet cells in the intestinal epithelium of mice, under experimental conditions indicating that this is a premitotic phenomenon rather than a post-mitotic inheritance from a parent cell.

It seems useless to attempt further analysis of these results until they can be extended and refined by additional experiments. It is suggested, however, that the regenerating pyloric caecum of asteroids, like the regenerating digestive system of holothuroids, provides excellent material for the study of cell proliferation and differentiation. The events in regeneration of the parietal parts of an autotomized ray, and the distribution of mitotic activity in regions other than those directly involved in caecal regeneration, are related subjects best reserved for discussion elsewhere.

SUMMARY

1. The regenerative replacement of excised pyloric caeca has been studied in three species of sea-stars belonging to the Family Asteriidae. Regenerating specimens have been observed, and the histological events of regeneration followed by serial sections, to the point at which essentially normal organs are again in place and functioning. The process of regeneration is practically identical in all, but it occurs at a much slower rate in *Leptasterias* and *Pisaster* than in *Asterias*. Rate differences are attributable to differences in environmental temperature.

2. Caecal regeneration involves a sequence of changes resembling those previously observed in *Henricia*. Mesenteric tunnels guide the advance of simple tubular outgrowths from the pyloric duct; bifurcation of the caecum occurs at the point where the tunnel forks, giving rise to paired tubes growing distally parallel with each other. Differentiation of epithelial elements, with functional secretory cells, proceeds close behind the advancing tip of each tube. More proximally, lateral expansions produce pockets in which gland cells become localized, while less highly-specialized current-producing cells line the roof and floor of the median duct. The gradient of differentiation extends from the young, undifferentiated growing tip to the oldest region at the base of the regenerate. The pyloric caecum growing inside a regenerating ray following induced autotomy forms through the same sequence of events found in replacement of an excised caecum. Comparison with descriptions of post-larval formation of the pyloric caeca in the developing sea-star reveals that regeneration follows the same course as original formation.

3. Isolated segments of partially extirpated organs are resorbed without producing regenerative growth; their presence in the mesenteric tunnel does not affect normal outgrowth from the central stump. *Pisaster* and *Asterias* are capable of regenerating all 5 pairs of pyloric caeca at once, at rates somewhat slower than those involved in single-pair replacement. The return of function in these caecum-

less animals has been charted by observation of feeding behavior and digestive capability.

4. The suggestion that growth of the replacement caecum occurs through mobilization and incorporation of amoebocytes, based on earlier failure to identify mitotic activity in *Henricia,* is now shown to be invalid. Autoradiographs made from sections of two-week regenerates in *Asterias* after injection of thymidine-H^3 reveal large numbers of cells in some phase of mitotic activity. Direct observation of mitotic figures in these same preparations confirms the fact that growth of the regenerating caecum involves mitotic proliferation. Cell division is most common among apparently undifferentiated cells with large, vesicular nuclei; it occurs where such cells occur, in both lining epithelium and covering peritoneum, at all levels of the advancing regenerate. In proximal regions, dividing cells are most numerous where the caecal walls are outfolding to form glandular pockets.

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NORMAL EMBRYONIC STAGES OF THE SQUID, LOLIGO PEALII (LESUEUR)[1, 2]

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The need for a series of normal developmental standards for any experimental embryo is obvious, but some justification for further burdening the literature with another species might be in order. Recently, *Loligo pealii* embryos have been shown to be quite useful experimental organisms (Arnold, 1961a, 1961b, 1963a, 1963b). The only other developmental series for any of the cephalopods known to the author was done by Naef (1928) in his extensive monograph on this class. While the quality of Naef's work is indisputable his stages do not include cleavage in the numbered series and in some cases experimentally significant developmental events are not separated by his 18 stages for *Loligo vulgaris*. Apparently, the major criterion used by Naef was chronological age by days of development rather than morphological events. This places some rather major events within the same stage (*e.g.*, all early cleavage stage I) and places abnormal significance on developmental rate. Furthermore, slight differences in the sequence of events occur when the development of *L. vulgaris* is compared with that of *L. pealii*. For these reasons it was felt that a comprehensive staging of *L. pealii* would be useful. Hopefully, the publication of such a staging would encourage further work on this embryo and serve as a teaching aid.

MATERIALS AND METHODS

The drawings which make up these stages were made under standardized conditions, to eliminate as much variation as possible. The embryos which were used were all of known chronological age and were kept in running sea water at 20 to 21° C. All of these eggs were laid in the laboratory under conditions previously described (Arnold, 1962). An egg string with embryos of uniform age was selected, carefully examined, and a representative embryo drawn with the use of a 32-power microscope and camera lucida. Details were added at a magnification of 100 power. After several such drawings had been made and compared, a preliminary staging was constructed and checked against living material. Two revisions of the preliminary staging were made with additions and corrections based on concurrent observations.

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[1] This work is a portion of a thesis submitted in partial fulfillment of the Ph.D. requirements of the Department of Zoology, University of Minnesota.

[2] Part of this work was done while the author was the recipient of an N.S.F. Predoctoral Fellowship.

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All of the drawings were made from living embryos, with the exception of stages 17 and 18. In these cases the outlines of the embryos were drawn from life and mercuric chloride was added to the sea water containing the embryos to make the cells more opaque. In this way it was possible to more easily observe the organ primordia and it was even possible to distinguish regions of columnar cells from those of cuboidal cells. However, with practice it is possible to make these same observations on living material. The later stages were immobilized with chloretone or by chilling.

In order to have these drawings comparable in size, 1800 embryos were measured in length and width. In the older embryos the distance across the eyes was used for the width measurement, and from stage 27 to stage 30 the distance from the posterior edge of the mantle to the base of the yolk sac was measured for the length measurement. These embryos were from at least three different egg strings which were laid by different females. The averages of these measurements were used to adjust photographically the relative sizes of the drawings so that the figures have the proper size relationship.

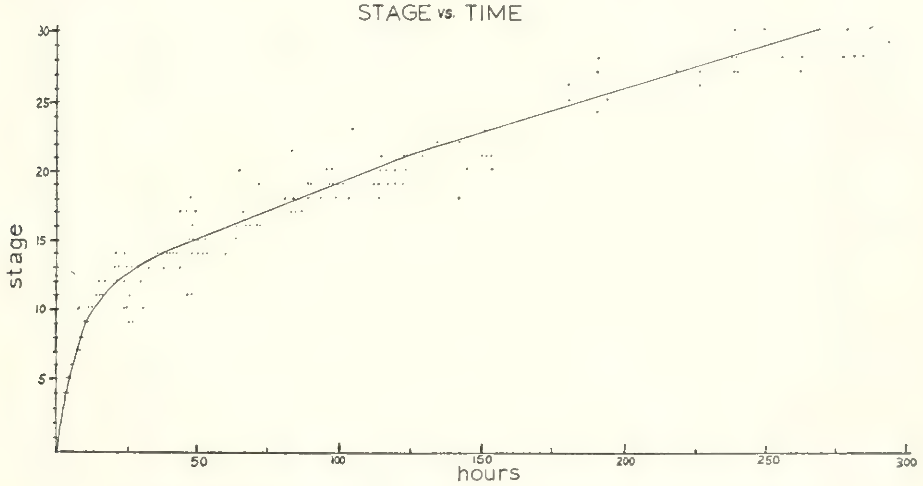


FIGURE 1. Chronology of development, based on 142 embryos observed from time of laying until stage indicated (20–21° C.).

A total of 142 observations was made on embryos laid at known times so that a chronology could be constructed (Fig. 1). The variability shown here obviates the use of chronological age alone for a criterion of development age. However, these data are presented because they should prove useful in approximation.

NORMAL STAGES OF DEVELOPMENT

The following developmental stages are solely based on morphological features, and a certain amount of heterochrony should be expected when these drawings are compared with living material. These stages have been successfully used with other genera (*Sepioteuthis, Octopus*) by the author although some of the criteria used here must be ignored. The identifying characteristics mentioned below are listed more or less in order of prominence and/or importance. A detailed description of morphological features will not be made here and the reader is referred to the general descriptions by Korschelt and Heider (1910, 1936) and MacBride (1914), as well as the more specific papers by Sacarrao (1945, 1952, 1953, 1954, 1956), Fioroni (1963), and von Orelli (1959) for an introduction to this literature. However, Figure 2 is included for the sake of convenience.

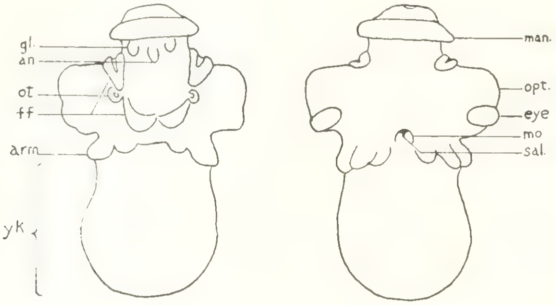
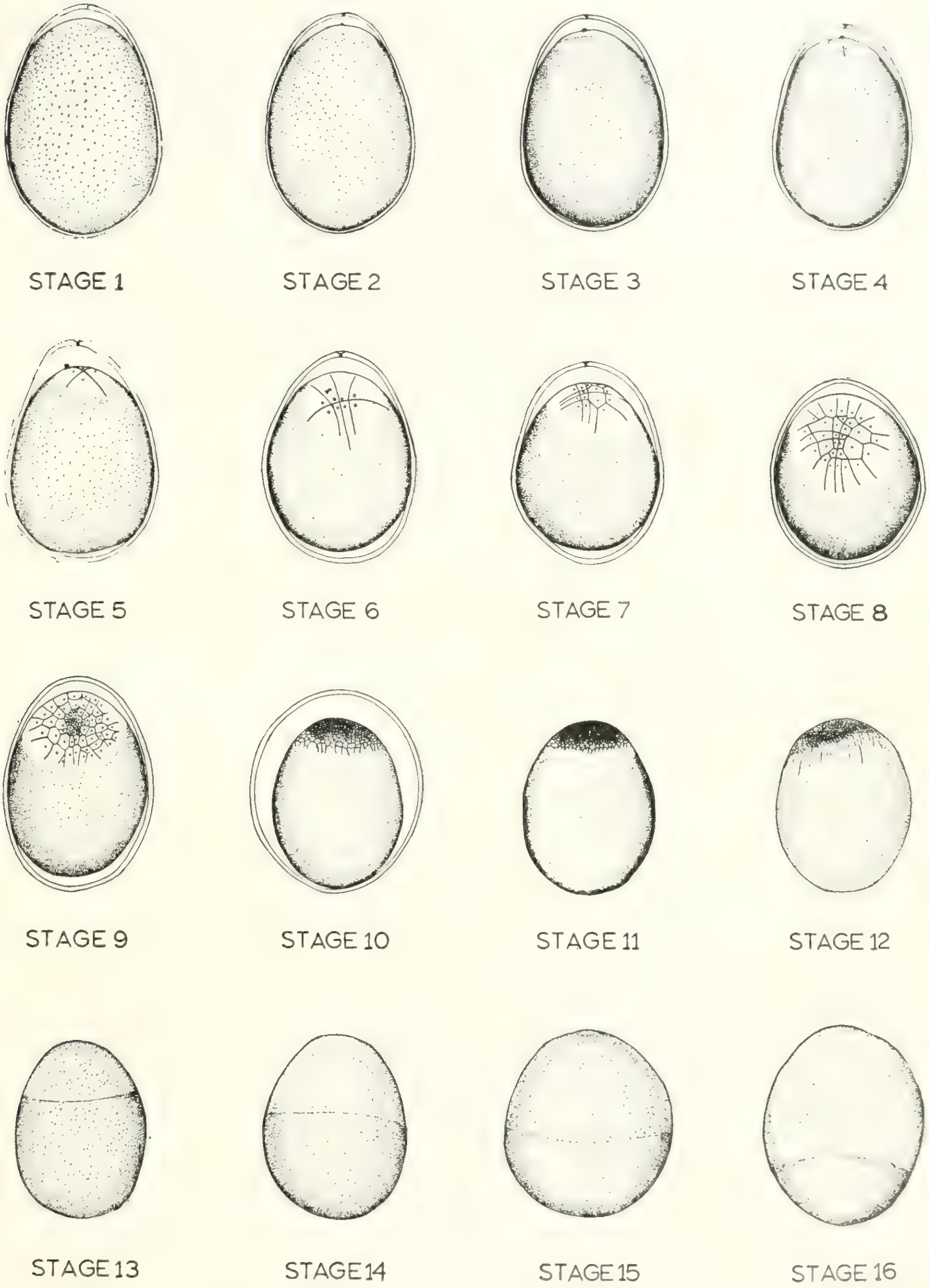


FIGURE 2. Organ *Anlagen* of embryo at about stage 21. Gl., gill; an., anal papilla; ot., otocyst; ff., funnel folds; yk., yolk sac; sh., shell gland; opt., optic ganglion; mo., mouth; sal., salivary pit.

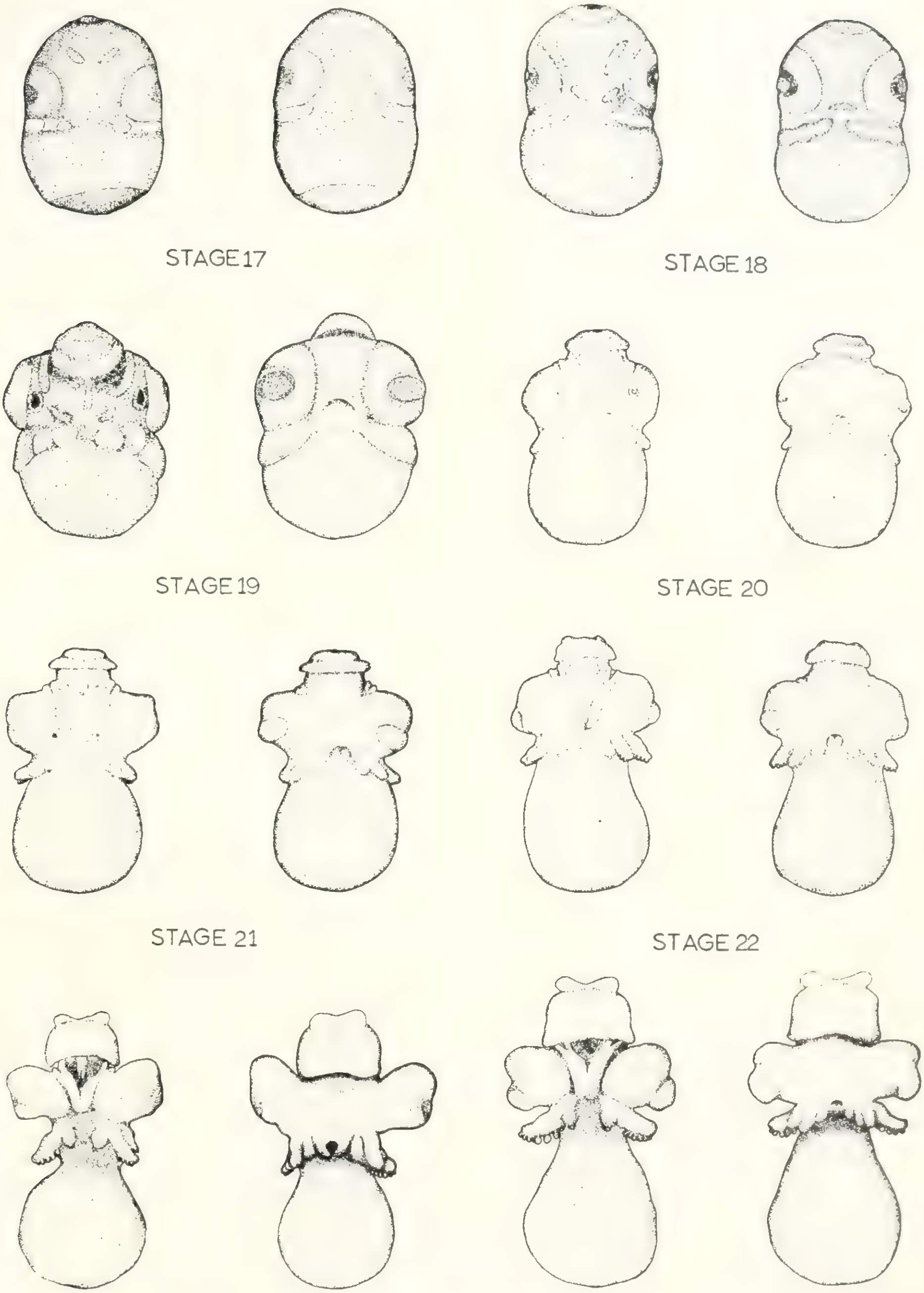
STAGE 1. Fertilized but lacking polar bodies. Ooplasmic streaming occurring to form the blastodisc.
STAGE 2. First maturation division. Blastodisc quite evident.
STAGE 3. Second maturation division. Slight increase in the size of the blastodisc.
STAGE 4. First cleavage along the plane of symmetry.
STAGE 5. Second cleavage occurs asymmetrically. Embryonic anterior and posterior are evident.
STAGE 6. Third cleavage. Division is unequal and asynchronous.
STAGE 7. Fourth cleavage. Division is unequal and asynchronous.
STAGE 8. Fifth cleavage. Blastoderm often asymmetrical and somewhat irregular. About 32 cells.
STAGE 9. Sixth cleavage. About 64 cells. Embryonic anterior and posterior no longer distinguishable.
STAGE 10. Formation of a second layer of cells to form a papilla of yolk in the center of the blastoderm.
STAGE 11. Yolk papilla flattened. Blastoderm spreading by marginal division.
STAGE 12. Blastocones can be distinguished but not easily in this species.
STAGE 13. Blastoderm covers about one-third of the egg. Blastocones no longer evident.
STAGE 14. About two-fifths of the egg cellulated.
STAGE 15. About three-fifths of the egg cellulated.



STAGE 1
STAGE 2
STAGE 3
STAGE 4
STAGE 5
STAGE 6
STAGE 7
STAGE 8
STAGE 9
STAGE 10
STAGE 11
STAGE 12
STAGE 13
STAGE 14
STAGE 15
STAGE 16

FIGURE 3. Stages 1 to 16. See text (ca. 24 ×).

STAGE 16.	About two-thirds or more of the egg cellulated. Shell gland first visible.
STAGE 17.	Major organ primordia evident as thickenings in the blastoderm. About five-sixths of the egg surface covered by cells.
STAGE 18.	Cellulation complete. Slight "waist" is visible. Mouth, anterior and posterior funnel folds present as thickened placodes. Invagination of the eyes just beginning.
STAGE 19.	Eyes and shell gland stand out slightly from the rest of the embryo. Mouth and eye invagination has begun. Arm primordia discrete. Otocysts present as placodes and slightly invaginated.
STAGE 20.	Gills, arms, funnel folds and mantle are elevated from the rest of the embryo. Eye invagination nearly complete. Salivary pit evident in the mouth. Shell gland invagination closing. Otocyst still has small opening to the exterior.
STAGE 21.	Eye vesicles completely closed and stand out on prominent stalks. Anterior funnel folds fused at margins. Invagination of the shell gland complete. Primordia of the optic lobes are evident as discrete masses of tissue. Sucker primordia just evident on the arms.
STAGE 22.	Mantle beginning to grow downward. Fins evident on the mantle. Distal edges of the anterior funnel folds prominently bent toward the midline. Anterior and posterior funnel folds fused together and continuous. Optic lobes prominent posterior to the eyes. Lens primordium may be seen with care.
STAGE 23.	Mantle covers about one-half of the gills. Funnel folds have formed the siphon at their anterior extremity. Yolk sac discretely separate from the embryo proper. Retina curved. Lens evident as a refractive rod. Iris folds have occurred.
STAGE 24.	Mantle completely covers the anal papilla and the gills, but the funnel muscles are still evident. Median margins of the funnel folds fusing. Mouth completed. Hearts beating.
STAGE 25.	Mantle covers the posterior portion of the funnel but a small triangular opening is evident. Median margin of fins just fused. Iris of eye prominent as a colored circle. Lens spherical. Individual gill filaments barely visible.
STAGE 26.	Mantle completely covers posterior margin of the funnel. Pigmentation has begun in the eyes and ventral chromatophores (orange). Individual filaments of the gills prominent.
STAGE 27.	Secondary cornea beginning to cover the eye. Eyes move freely in the orbit. Dorsal chromatophores evident but relatively few in number.
STAGE 28.	Secondary cornea completely covers the eyes. Organ of Hoyle begins to be prominent. Yolk sac approximately same size as the head.
STAGE 29.	Pigmentation of ink sac occurs. Organ of Hoyle quite evident. Arms equal in length to yolk sac. Hatching may occur.
STAGE 30.	Hatching and loss of yolk sac. Organ of Hoyle depleted.



STAGE 17

STAGE 18

STAGE 19

STAGE 20

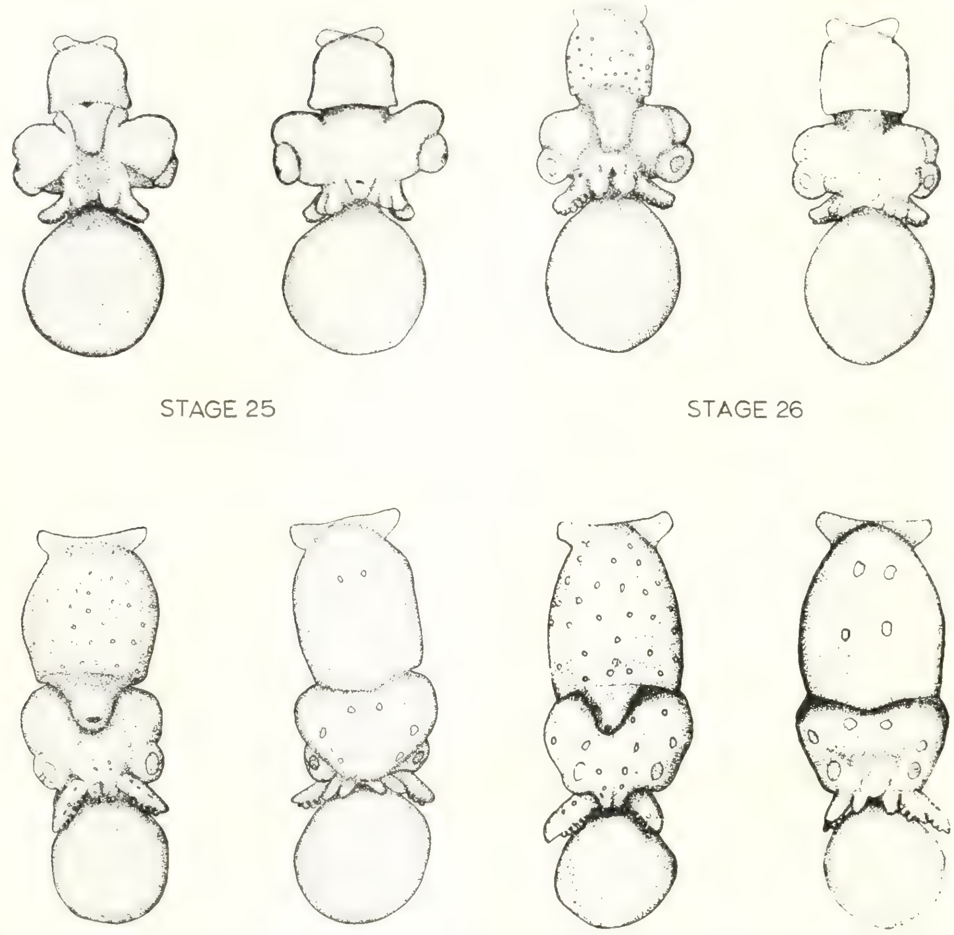
STAGE 21

STAGE 22

STAGE 23

STAGE 24

FIGURE 4. Stages 17 to 24. See text (ca. 24 ×).



STAGE 25

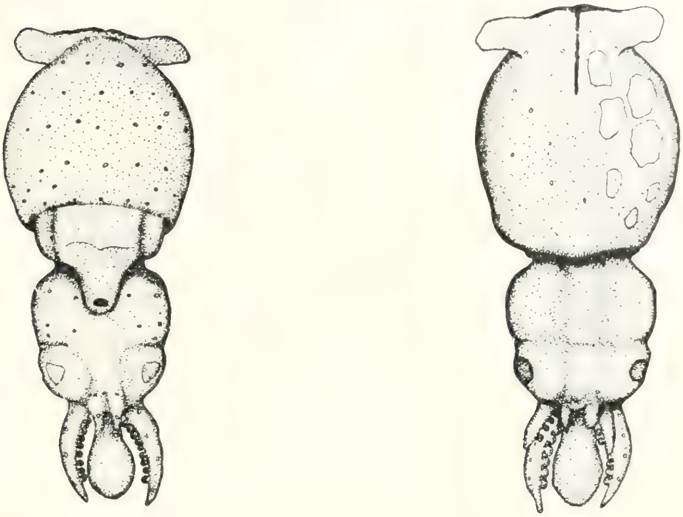
STAGE 26

STAGE 27

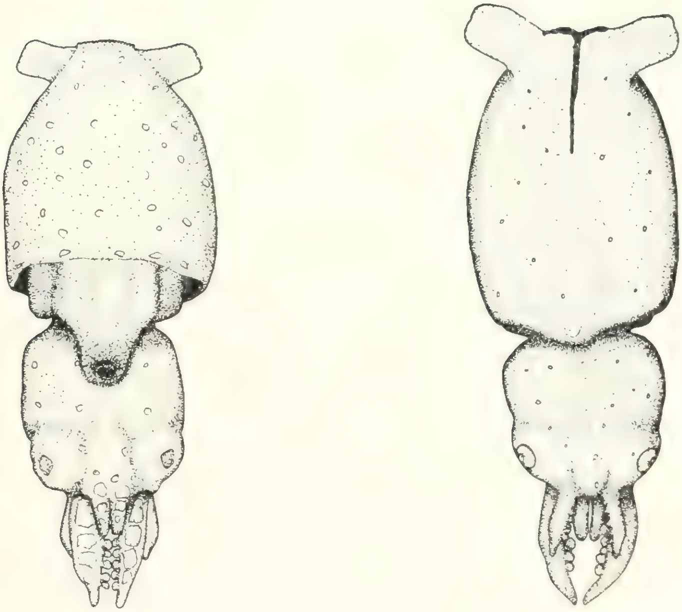
STAGE 28

FIGURE 5. Stages 25 to 28. See text (ca. 24 ×).

The author feels that these 30 stages represent relatively discrete and easily identifiable stages in the development of *L. pealii*. However, each stage is artificial and should be regarded as an interval in a time sequence rather than a distinct step to be replaced by another. Naturally, some overlap between stages occurs but by using several criteria the developmental age of any embryo can be fairly accurately determined. For this purpose these stages can also be described as "early" or "late," depending on just how far the developmental criteria have proceeded. As with other organisms, variation between individuals and between batches of eggs must be expected. Hopefully, the criteria used here have a relatively low amount of variability but some criteria are more reliable than others. An example of this is hatching. Normally hatching occurs when all of the yolk has been



STAGE 29



STAGE 30

Figure 6. Stages 29 and 30. See text (ca. 24 ×).

consumed or dropped from the larva. Frequently hatching will occur in stage 29 if the egg strings are mechanically disturbed. In using these stages there is no substitute for experience and familiarity with the living material.

I would like to thank Dr. Sidney B. Simpson, Jr., for reading this manuscript.

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AN ANGLE SENSE IN THE ORIENTATION OF A MILLIPEDE[1]

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The literature of experimental psychology contains numerous studies of a maze-running phenomenon which has been called "reverse turning." This term refers to the subsequent reaction of an animal which has been forced to make a right-angle turn in a maze immediately before encountering a free choice point where it can turn to right or left. At the choice point an animal exhibiting reverse turning, turns to the side opposite that of the preceding forced turn; for instance, a forced left turn is followed by choice of a right turn. This alternation of turning direction has been reported for a variety of organisms throughout the animal kingdom. The reasons for studying the phenomenon have been diverse and have led to several different interpretations. It seems worthwhile, since it has not been done before, to review the different approaches to the problem.

The first extensive examination of reverse turning in a maze was made by Schneirla (1929) in his studies of learning in ants. He found a strong tendency for two species of *Formica* to alternate turning directions at a choice point immediately following a forced right-angle turn. Schneirla was interested in the phenomenon from the standpoint of maze design for the study of learning. The choice at a junction within the maze could be strongly influenced by the pattern of the preceding turns. If the reverse turning tendency were operating and the choice it favored were a blind alley, turning into this alley would be eliminated only with great difficulty during learning of the correct maze pathway. Schneirla explained reverse turning by "centrifugal swing," in which he distinguished two components, the first being the effect of momentum and the second, a thigmotactic response. According to his interpretation, the running ant, upon entering a right-angle turn, would be carried by its momentum into contact with the outside wall of the turn; subsequently the ant would follow this wall and, at the free choice point, would tend to turn toward the side of antennal contact.

Soon afterward, reverse turning was reported in the maze running of white rats by Dashiell and Bayroff (1931). During maze running in rats, these authors did not observe any obvious bodily displacements which corresponded to those upon which the centrifugal swing theory was based. To account for the reverse turning they proposed that "the factor most responsible is a forward-going tendency in animal locomotion that leads not only to maintenance for short distances of a direction already set but also to a compensatory sort of correction when forced out of line by an obstruction" (p. 94).

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Following the report of Dashiell and Bayroff (1931) there appeared a number of papers (Schneirla, 1933; Ballachey and Buell, 1934; Witkin and Schneirla, 1937) which attempted to demonstrate that the reverse turning of rats could be accounted for by the centrifugal swing principle and that it was unnecessary to invoke any innate "forward-going tendency." Apparently these arguments for centrifugal swing were not wholly convincing. While acknowledging the important role of mechanical inertia and displacement in maze performance, Warden, Jenkins and Warner (1940, p. 805) wrote, "A more likely interpretation of the 'reverse turn' subsequently displayed at the junction, however, is that the phenomenon is primarily the *direct* outcome of behavioral inertia (postural activity) based in part on stimulation of tension receptors through inertial changes precipitated at the previous turn and in part on the set of the organism antecedent to the previous turn."

In 1948 Hullo observed reverse turning in the cockroach *Blatella germanica* run on an elevated maze. The turning was attributed to centrifugal swing.

In the 1950s there was a renewal of interest in reverse turning. Demonstrations of its occurrence in several organisms were presented as evidence for Hull's principle of reactive inhibition. Hull stated the principle as follows (1943, p. 300): "Whenever a reaction is evoked in an organism there is created as a result a primary negative drive; this has an innate capacity to inhibit the reaction potentiality to that response; the amount of net inhibition generated by a sequence of reaction evocations is a simple linear increasing function of the number of evocations; and it is a positively accelerated increasing function of the work involved in the execution of the response; reactive inhibition spontaneously dissipates as a simple negative growth function of time." Presumably, then, in the maze situation there would occur, following a forced turn to the right, a temporary inhibition of the right-turning tendency, and at the next choice point the animal would turn left. As support for this concept Lepley and Rice (1952) reported that *Paramecium multimicronucleatum* in a microscopic maze tended to alternate its turning direction following a forced turn. However, Jensen (1959) pointed out that centrifugal swing had not been controlled in these experiments, and that this latter alternative seemed the more plausible explanation. Furthermore, using *Paramecium caudatum*, Lachman and Havlena (1962) were unable to reproduce the results of Lepley and Rice. Perhaps it should be noted that this experiment differed from the experiment of Lepley and Rice in that the choice occurred at a Y rather than a T junction. This point is mentioned because it has been suggested that "invertebrates" may not alternate turning directions at a choice point in a Y-maze (Hayes and Warren, 1963). Apparently this generalization was based on the report that the beetle, *Aleochara bilineata,* did not exhibit spontaneous alternation (see below) in a Y-maze (Putnam, 1962). Even in this case, however, turning of the beetle in the maze was not random. Instead of alternating, the beetle showed a highly significant tendency to repeat its preceding choice.

Mealworms, larvae of *Tenebrio molitor,* were found to alternate turning directions after a forced turn (Grosslight and Ticknor, 1953). The tendency to alternate was greater following two consecutive turns in the same direction and decreased when the distance between forced turn and choice point was increased. Following Jensen's (1959) criticism that centrifugal swing, in particular the thigmotactic component, had not been controlled, the experiment was repeated with a narrow maze in which the mealworm was presumably equally stimulated on both sides of its body at the choice point (Grosslight and Harrison, 1961). The authors found that even with thigmotaxis controlled, the reverse turning tendency persisted with the same intensity found in the earlier study. They interpreted their results in terms of reactive inhibition.

Dingle (1964b) has confirmed the occurrence of reverse turning in *Tenebrio* larvae, but he has made additional observations which he believes are incompatible with the principles of reactive inhibition, as originally formulated by Hull (1943). First, using a maze which presented the three alternatives of turning right or left or continuing straight ahead, he found that following a forced turn to the right, no animals chose to turn right and the number turning left was slightly greater than the number continuing straight ahead. Control animals not subjected to forced turning chose to continue straight ahead. Thus, the strong tendency for left turning following forced right turning reflected not simply inhibition of right turning, but rather a tendency to counteract specifically the preceding forced right turn. Second, reverse turning increased as the distance between starting point and forced turn was increased. Thus, the intensity of reverse turning in *Tenebrio* was dependent upon events occurring prior to the forced turn. Third, when the distance between forced turn and choice point was increased from four to eight centimeters, reverse turning disappeared and most larvae chose to continue straight ahead. However, when larvae were retained under cotton wool immediately following the forced turn, for a period of time necessary to crawl eight centimeters, and then were permitted to crawl four centimeters to the choice point, they evinced strong reverse turning. Thus, within the limits studied the decay of reverse turning tendency was not a function of time but rather of distance crawled. For these reasons Dingle rejects reactive inhibition as an explanation of reverse turning in *Tenebrio*.

The terrestrial isopod, *Armadillidium vulgare*, was reported to alternate its turning directions with high significance at successive choice points in a maze (Watanabe and Iwata, 1956). Reverse turning tendency was greater following two consecutive turns toward the same side and decreased as distance between the choice point and preceding turn increased. Results were interpreted in terms of reactive inhibition. The authors did not consider the possibility that their results could be explained by centrifugal swing.

Rice and Lawless (1957) found no evidence for reverse turning in planarians at a choice point following a forced turn. The possible influence of thigmotaxis was eliminated by discarding all worms which followed the wall of the maze after making the forced turn. Of course, this criterion would eliminate all worms which had attempted to alternate their turning direction following the forced turn but before reaching the choice point and, as a consequence, had come into contact with the outside wall.

Dingle (1961a, 1962) has reported that the boxelder bug, *Leptocoris trivittatus*, and several other insects, when forced to make a right-angle turn on an elevated maze, tended to turn in the opposite direction rather than continue straight ahead. In *Leptocoris* the tendency to alternate turning directions increased both when the distance between starting point and forced turn was increased and when the distance between forced turn and choice point was decreased. Dingle suggested that reverse turning is an adaptive behavior peculiar to insects living on the outer leaves of bushes and branches. He interpreted the response essentially as a manifestation of a forward-going tendency. Like Warden, Jenkins and Warner (1940), Dingle believed that reverse turning was an attempt by the organism to re-establish a "set" which had been created by sensory inflow before encountering the forced turn. In these first experiments with boxelder bugs an open window served as the light source. Dingle performed control experiments in order to eliminate the possibility that phototaxis was the basis for reverse turning. Another possibility not discussed by him was that rather than being a simple phototaxis, the response was a menotaxis, or compass reaction, in which the bug moved at a fixed angle to the light source. This explanation would be compatible with Dingle's analysis of the response. Thus, the "set" established prior to the forced turn would be the bug's orientation relative to the asymmetrical light field. Following the forced turn the bug would temporarily attempt to regain its original orientation or "set" until it assumed its newly imposed one. A concurrent report by Dingle (1961b) that the reverse turning tendency was reduced as the intensity of illumination was reduced indicated that vision indeed was involved in the response.

In a recent paper Dingle (1964a) has reported additional characteristics of the reverse turning tendency in boxelder bugs. The tendency was present to a small but statistically significant extent in blinded bugs. Both normal and blinded bugs exhibited the tendency following forced runs on curved causeways; the degree of reverse turning was increased as the distance run was increased and as the radius of curvature of the causeway was decreased. Dingle was able to quantify the turning tendency by substituting a level platform for the choice point; upon the platform and about the point of emergence from maze pathway onto platform were inscribed arcs divided into 10° sectors. With this apparatus he was able to describe in terms of angular turning tendency the effects both of forced runs on curved causeways and of different starting point to forced turn distances. Also, he indicated that Akre (1962), using mazes similar to his own, had found reverse turning in the red milkweed bug, *Tetraopes tetraophthalmus.*

A different approach to turning reactions has employed the simple T-maze. The turning phenomenon observed in the T-maze is referred to as "spontaneous alternation." In this maze the animal is required to make right-angle turns at the junction of the T on successive trials. On the initial trial the animal may be either permitted a free choice or forced to enter one of the arms. It is then returned to the starting arm, and its choice on the second trial is recorded as a repeat or alternation of the initial choice. White rats, which have been studied most extensively in this maze, display a strong tendency to alternate.

Use of the T-maze eliminates the mechanical components of centrifugal swing, momentum and the subsequent contact response. However, a large body of work, reviewed by Dember and Fowler (1958), has demonstrated that the turning response in such a maze is in fact very complex. When a rat is subjected to a second trial in the same maze it is exposed to the same places and stimuli already encountered on the preceding trial. With the introduction of these variables, it is possible that the rat may alternate its turning with respect to one or a combination of factors. The following are two examples of interpretations which have been suggested at various times for spontaneous alternation. First, alternation may be the result of a persisting negative reaction to the turning response of the preceding trial, as the proponents of reactive inhibition have suggested. Thus, spontaneous alternation and reverse turning would have the same basis, that is, alternation of turning with respect to an immediately preceding *response*. This theory, however, has been found to be inadequate for explaining a number of aspects of spontaneous alternation. Second, alternation may occur with respect to the *place* and *stimuli* encountered on the preceding run. In line with this second viewpoint, spontaneous alternation has been interpreted in terms of exploratory drive, curiosity, and stimulus satiation, and it is upon these factors that recent psychological research has focused.

A third explanation for spontaneous alternation, which has not been considered by alternation theorists, is that under certain circumstances spontaneous alternation may reflect the forward-going tendency proposed for rats by Dashiell and Bayroff (1931). Alternation on the second trial would result from a persisting tendency to correct for the forced turn experienced on the initial trial. This explanation is similar to reactive inhibition because it states that spontaneous alternation and reverse turning are each manifestations of the same behavioral tendency and both are reactions to the preceding forced response. Although it is now established that psychological factors, such as curiosity, may be important determinants of spontaneous alternation, this third explanation is advanced because it may be significant in maze situations, and in organisms other than white rats, where the psychological factors are not dominant or even involved.

One of the rat experiments possibly germane to the experiments to be described herein is the report of a spatial gradient in alternation tendency (Zeaman and Angell, 1953). Rats were run on a fan-shaped elevated maze consisting of choice alleys located at 90° to the right, 30° to the right, 30° to the left, and 90° to the left of the starting arm. The rats were first forced for either two or ten runs onto one of the 90° alleys and were then given a free-choice trial in which all alleys were open. There was a tendency to choose with increasing frequency the alleys farther removed from the original forced alley. The gradient of responses was more pronounced after ten forced trials than after two forced trials.

Spontaneous alternation has been reported in earthworms. *Lumbricus terrestris* was found to alternate on successive trials in a T-maze (Wayner and Zellner, 1958). When the suprapharyngeal ganglion was removed, the tendency to alternate was reduced and in some worms was replaced by a tendency to repeat the preceding choice. In a recent review Jacobson (1963) describes the following additional unpublished experiments on alternation in annelids. Arbit and McLean (1959) employed the same technique used with rats by Zeaman and Angell (1953) but failed to find a spatial gradient in alternation tendency in *Lumbricus*. Fraser (1958) found that the earthworm, *Allolobophora terrestris longa*, alternated turns in a T-maze significantly more often than chance expectation, and the earthworm, *Lumbricus rubellus*, alternated significantly less often. Kasper (1961) has reported that an earthworm can exhibit reverse turning in a maze pathway consisting of a forced turn followed by a free choice point.

Iwahara (1956) found no evidence for spontaneous alternation in cockroaches run in a Y-maze with inter-trial intervals of either 20 or 120 seconds.

From the foregoing review it is evident that in a number of experiments there was failure to control for such well established behavioral responses as thigmotaxis, phototaxis, menotaxis, and in some cases, possibly, the following of chemical trails. Perhaps part of the reason for this failure is that many authors have regarded reverse turning and spontaneous alternation primarily as maze phenomena and have not recognized their possible biological significance as important components of animal orientation reactions. The further possibility that reverse turning represents a kinesthetic response was suggested more than twenty years ago, and good evidence for it has been provided by recent experiments. It is notable that two reviewers of animal orientation, Lindauer (1963) and Jander (1963), have considered the experiments on reverse turning in mealworms (Grosslight and Harrison, 1961) and boxelder bugs (Dingle, 1961a) as examples of kinesthetic orientation. For boxelder bugs it has been pointed out that more convincing evidence for a kinesthetic contribution was actually provided by later experiments in which visual cues were eliminated by blinding the bugs (Dingle, 1964a).

The following experiments demonstrate a reverse turning tendency in another group, the Diplopoda. It has been possible to show that a millipede species is capable of precise quantitative compensation for forced turning through a large range of angles from an initial path.

Materials and Methods

Tropical millipedes, *Trigoniulus lumbricinus* (Gerstaecker 1873), were ideal for experimental use. They were quite tractable, crawled about actively when handled, and did not appear to fatigue during an experiment. Generally they were collected on the day of the experiment from leaf litter beneath a breadfruit tree in the garden of the Museu Goeldi in Belém, Brazil, where all experiments were performed. At least one-half hour before being tested, each animal was placed in the dimly illuminated orientation chamber for a period of light adaptation. The animals used were approximately 4 mm. in width and ranged from 43 to 54 mm. in length.

The object of experimental manipulation was to force the millipede to turn from an initial path through a specified angle and to measure any subsequent orientative response to the forced turning. This was accomplished by causing the animal to crawl through a narrow corridor, in the middle of which was an abrupt turn. When the animal emerged from the corridor, its amount of right or left turning was measured in terms of degrees of angular deviation from a straight-ahead path. One of the corridors used is illustrated in Figure 1. A series of such corridors was constructed of smooth wooden blocks glued to sheets of graph paper. The dimensions of all corridors were the same: total length, 14 cm.; height, 7 mm.; and width, 6 mm. Only the angle of the forced turn was varied. For measuring the angle of emergence an arc was circumscribed on the graph paper at a distance of 6.35 cm. from the exit and divided into 5° sectors. The animal's orientation was recorded as the sector in which its head first reached the arc. A transparent glass plate served to cover the corridor. Dingle (1964a) has described a similar method for quantifying the reverse turning tendency in boxelder bugs.

The length of each of the three components of the experimental path, corridor entrance to turn (7 cm.), turn to corridor exit (7 cm.), and corridor exit to arc (6.35 cm.), exceeded the length of any animal used. Consequently, each animal was required to enter the corridor completely before encountering the turn, to straighten out completely between forced turn and exit, and to crawl a distance greater than its length from corridor exit to arc. Also, the corridor was sufficiently wide to permit the animals to emerge without touching either wall.

The visual environment of the millipedes was controlled by placing the corridor in use within a large chamber, which was 49 cm. high, 79 cm. wide, and 62 cm. long. An opening 30 cm. wide in the back of the chamber behind the corridor permitted observation and access for handling the animals. The interior of the chamber was painted mat black. It was dimly illuminated through a small hole, in the center of the top of the chamber, which was 1.5 cm. in diameter and covered with a white diffusing transmitter. The light source was a 25-watt frosted incandescent bulb centered 1.5 cm. above the small opening. During all experiments the exit of the corridor was directed southward and was centered directly under the light spot. Thus, at the instant of the animal's emergence from the corridor, the overhead light source itself could not serve as a horizontal directional reference cue. The experiments were always performed in a darkened room.

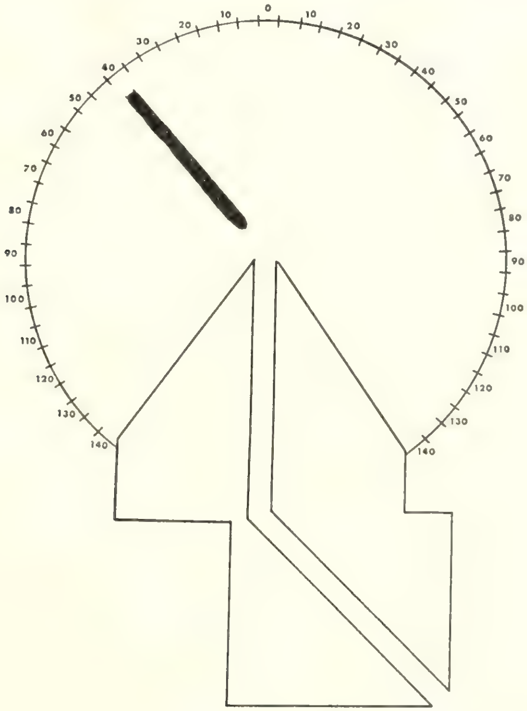

FIGURE 1. Orientation apparatus for quantifying reverse turning in millipedes. A millipede is depicted as responding to a 45° forced turn.

The experiments consisted of measuring the response of individuals to each of a series of angular turns. Two different series were used. The first, which will be referred to as Series I, consisted of forced right-hand turns and comprised the following angles: 0° (no turn), 15°, 30°, 45°, 60°, 75°, 90°, 105°, and 120°. Between Nov. 15 and Dec. 2, 1962, 45 animals were run through this series at various times of day from 7 AM to 11 PM. The average time required to complete an entire experimental series with a single animal was 29 minutes. The second series, designated Series II, comprised the following seven angles, ranging from 30° to the right to 30° to the left: 30°, 20°, 10° to the right; 0°; 10°, 20°, and 30° to the left. Between Nov. 29 and Dec. 11, 1962, 50 animals were run through this series. The average time required for a single animal was 17 minutes. In both series the response to each angle for each animal was taken as the average of five consecutive trials.

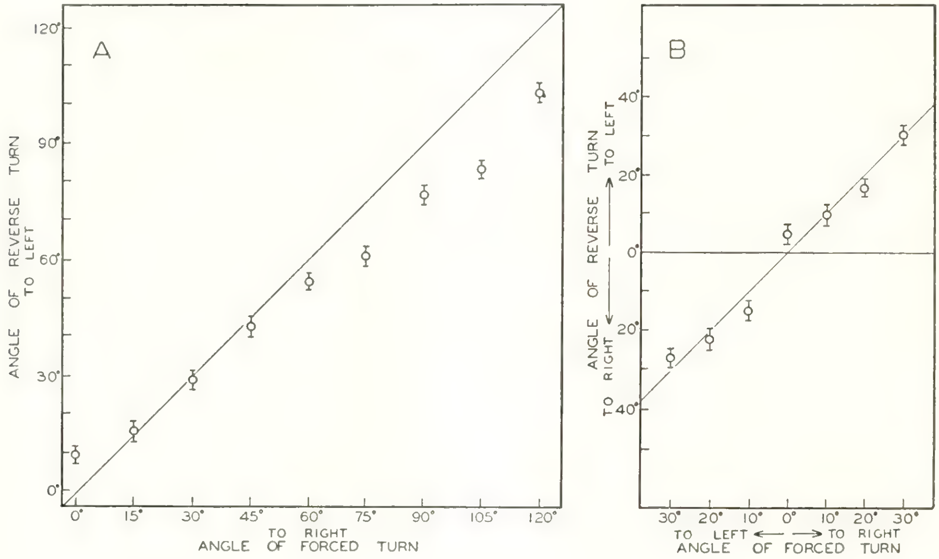

FIGURE 2. A. Mean angular turning response of millipedes to the forced angles of Series I. Standard errors of the mean are indicated. Diagonal line has a slope of 1. B. Same as A, but for Series II.

The procedure was the following. First, the animal's longitudinal axis was carefully aligned in front of the corridor entrance. The animal then was permitted to crawl through the corridor and across the arc. The orientation was recorded, and the animal was brushed lightly back to the corridor entrance where it was realigned and permitted to run again. When five runs had been completed the corridor was replaced by one with a different forced angle. The sequence in which the angles were presented to each animal was a random one, having been determined in advance by lottery.

Statistical formulas used in the analysis of data were those given by Walker and Lev (1953).

RESULTS

Results of the two experimental series are presented in Figure 2 and Table I. It is obvious that upon emerging from the corridor the millipedes turned on the average at an angle which was opposite, and in most cases approximately equal, to the angle of the forced turn. Clearly, the millipedes were alternating their turning quantitatively in a precise manner. That the precision of the response was essentially uniform over a wide range of forced angles was indicated by the similarity of variances for all angles (Table I). Comparison of mean values for the two independent series, I and II, revealed that the means for responses to the same angle, 30° to the right, were similar. Means for responses to intermediate angles in Series II, 20° and 10° right, were intermediate, although each of these two angles was not significantly different from means of responses to both of the forced

TABLE I

Means, variances and differences between means of responses by millipedes to angular forced turns

Angle of forced turn	Mean (degrees)	Variance (degrees)	Difference between adjacent means (degrees)	Probability (two-tailed t test of differences)
Series I				
0°	9.49	216.21		
			> 6.02	.06
15° R	15.51	279.53		
			>13.33	<.001
30° R	28.84	259.23		
			>13.69	<.001
45° R	42.53	295.85		
			>11.60	<.001
60° R	54.13	232.06		
			> 6.87	<.005
75° R	61.00	301.11		
			>15.90	<.001
90° R	76.90	267.01		
			> 6.53	<.005
105° R	83.43	280.16		
			>20.20	<.001
120° R	103.63	326.40		
Series II				
30° R	30.48	302.95		
			>13.82	<.001
20° R	16.66	200.56		
			> 6.82	<.005
10° R	9.84	346.74		
			> 5.28	.02
0°	4.56	293.90		
			>20.12	<.001
10° L	−15.56	296.82		
			> 7.22	<.02
20° L	−22.78	394.71		
			> 4.90	.07
30° L	−27.68	251.24		

angles in Series I which bracketed each of them. In both series the 0° response was asymmetrical, being twice as far to the left in Series I as in Series II.

In Series I the relationship between stimulus intensity and response intensity was not linear throughout the entire range of forced angles. From 15° to about 60° the angle of emergence was equal to the angle of the forced turn; above 60° the average angular turning upon emergence for all experiments was significantly less than the angle of the forced turn. For Series II the average values for all seven angles fell close to the regression line described by the equation $Y = X$, which indicates equivalence of stimulus and response. The computed least squares regression equation, $Y = 0.6° + 0.98X$, is scarcely distinguishable from this theoretical relationship.

Table II

Analysis of variance for comparing the turning responses of individuals to a series of angular forced turns

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Series I				
Total group	473,799.2	404		
Between means of individuals	43,704.1	44	993.28	5.427*
Between means of forced turns	365,666.8	8	45,708.35	249.731*
Error	64,428.3	352	183.03	
Series II				
Total group	238,664.6	349		
Between means of individuals	49,733.7	49	1,015.0	6.416*
Between means of forced turns	142,428.1	6	23,738.0	150.051*
Error	46,502.8	294	158.2	

* $P < 0.001$.

Measurements for all angles in a series were made on every individual. Therefore, it was possible to test with an analysis of variance for the significance of differences both in response to forced angles and in the response of individuals. In each series both responses to forced turns, as was evident from inspection, and the individual responses were highly significantly different from chance expectation (Table II).

A more detailed examination of the differences in response to forced angles was made by testing the significance of the difference between the means of responses to adjacent forced angles in each series. Comparisons were made with a t test in which the data were treated as non-independent samples in order to eliminate variation due to individual differences. For each pair of forced angles the significance of the mean of a population of differences between two measures on each individual was tested. Hence, the probabilities given in Table I for differences between means were not based on the variances listed in the neighboring column. In Series I, where 15° intervals separated adjacent forced turns, the means for all adjacent angles were highly significantly different with the exception of the difference between 15° and the asymmetrical 0° response (Table I). In Series II, where 10° intervals separated the forced angles, the probabilities were not so uniformly high as in Series I. Nevertheless, it appeared that on a statistical basis the millipedes were capable of distinguishing 10° differences in forced angles, even when the forced angle itself was only slightly larger than 0°.

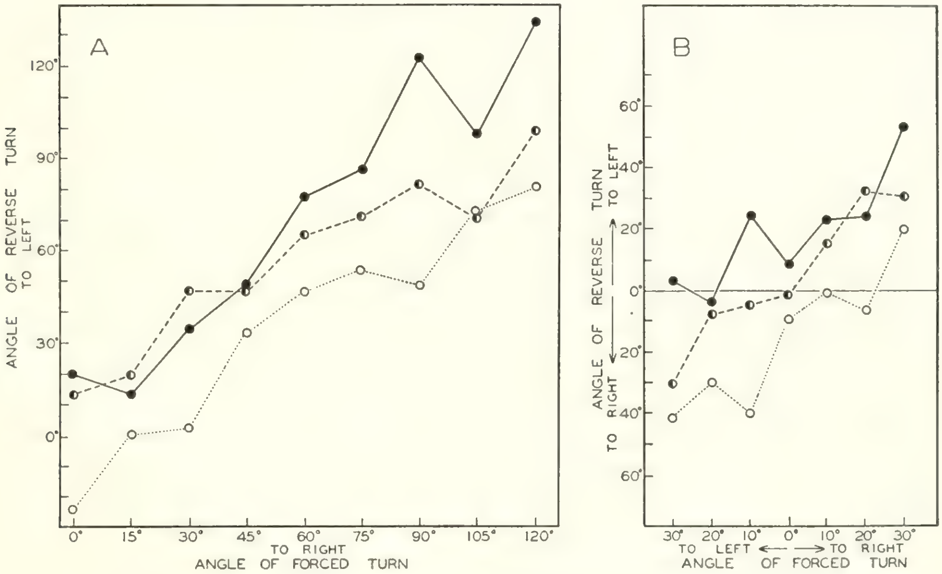

FIGURE 3. A. Mean angular turning response to forced angles of Series I for three millipedes selected from six run on Nov. 19, 1962. B. Same as A, for three millipedes selected from seven run on Dec. 1, 1962, in Series II.

The extent of the highly significant individual variation is indicated in Figure 3 by selected examples from each of the experimental series. Figure 3A represents the values for three individuals out of six which were run through Series I on Nov. 19. Figure 3B represents three out of seven individuals run through Series II on Dec. 1. There is a slight suggestion that the regression equation describing responses to a series of forced angles may differ in slope as well as intercept among individuals. The range of variation illustrated here is typical of that encountered among individuals on other days.

There is an additional large component of the variance in Series I which was not accounted for in the preceding analysis of variance. This variance is associated with the order in which the nine different forced angles were presented within individual series. Since this order was shuffled in every case, each of the forced angles happened by chance to be presented at least one in each of the positions

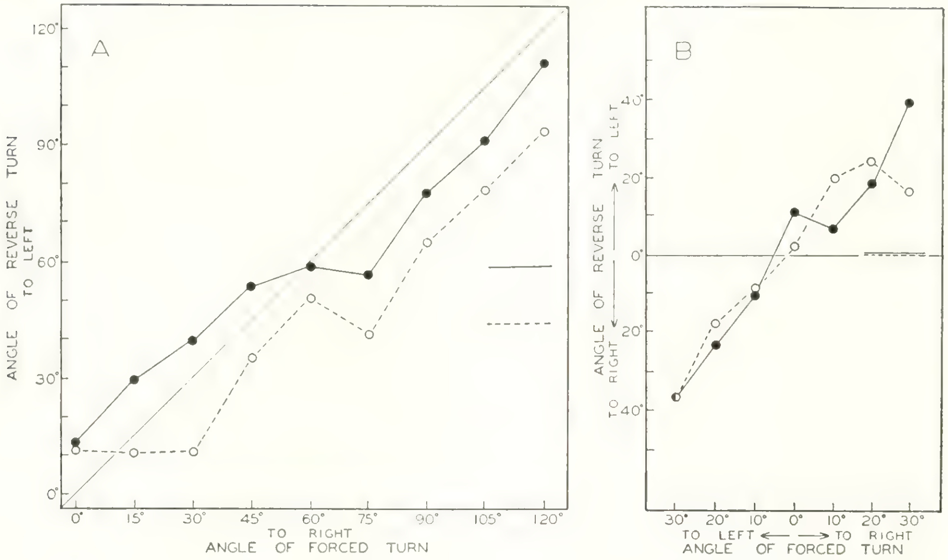

Figure 4. A. Mean angular turning response for all millipedes in Series I run first in sequence (open circles, dashed line) and ninth in sequence (closed circles, solid line). Mean values for each of the two series are indicated by horizontal lines to the right in the figure. Diagonal line has a slope of 1. B. Same as A, but for Series II.

from first through ninth. There was, however, one exception to this statement. The 15° forced turn never occurred fifth in any series. For the analysis to be presented in Figure 5A a value was interpolated for the response to this forced angle by taking the average of the mean responses to the 0° and 30° forced turns occurring fifth in sequence. The extent to which the response was affected by sequence position is shown in Figure 4A where the mean value for each forced

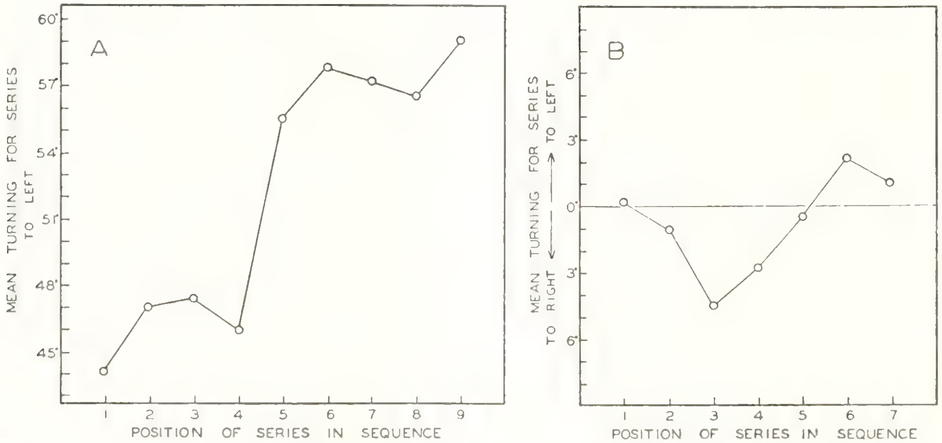

Figure 5. A. Mean angular turning for series occurring in each position in sequence in Series I. B. Same as A, but for Series II.

angle presented first in sequence is compared with the mean value when each angle was presented ninth, or last, in sequence. For all forced angles the response to the angle when it occurred ninth in order was greater than, or to the left of, the response to the same angle when it occurred first. The difference was largest at 15° and 30°, where it appeared that the animals did not distinguish among forced turns of 0°, 15°, and 30° on the first trial. To the right in Figure 4A two lines indicate the average difference between means for all nine angular forced turns in the two positions, first and ninth. The difference is 15°. The progressive character of the increase in turning tendency is shown in Figure 5A where the means for the angles of Series I occurring in each of the orders of first through ninth are graphed. These averages include the responses to the 0° corridor, although for this one angle there was no obvious trend toward displacement of the response to the left as one progressed through the nine orders of sequence. Values initially were low, increased rapidly between fourth and fifth positions and tended to level off during later positions.

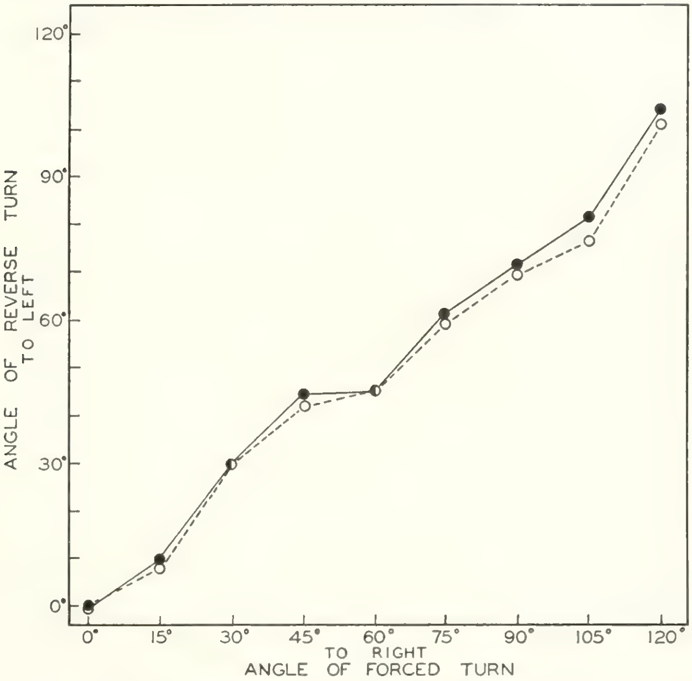

FIGURE 6. Mean angular turning response to forced angles for the last seven millipedes in Series I measured at two distances from the corridor exit, 6.35 cm. (open circles) and 10.2 cm. (closed circles).

When the data for Series II were subjected to the same analysis, no consistent differences were found (Figs. 4B and 5B). It is evident, however, that animals were responding on the first trial to forced angles of 30° and less.

As it emerged from the corridor the millipede oriented sharply and crawled off on a fairly straight course; the animal showed no obvious tendency to circle in either direction. In order to determine the directness and stability of the orientation, a second, concentric arc was traced on the orienting surface at a distance of 10.2 cm. from the exit. Comparison of the orientation angles of each millipede at two distances from the corridor exit, 6.35 and 10.2 cm., would disclose any tendency in the animal for continued turning after it had crossed the first arc. This additional measurement was made on the last seven millipedes run in Series I. The results are shown in Figure 6. It is evident that essentially all turning had been completed before the animal reached the first arc at 6.35 cm. from the exit. Dingle (1964a) has reported a slight but not statistically significant tendency for boxelder bugs to continue turning after making the initial reverse turning response. In millipedes the tendency to continue turning is much less than it is in boxelder bugs for turning responses of comparable size, 55° and less.

Discussion

In this study the millipede, *Trigoniulus lumbricinus,* was found capable of executing a reverse turn which was, on the average, precisely related to the angle of a preceding forced turn. The animal, therefore, appeared to possess an accurate angle sense. Furthermore, since the reverse turning response was delayed while the animal was forced to straighten out completely in a narrow corridor, the animal necessarily possessed a memory for the forced angle.

With the type of orientative apparatus used, the possibility that thigmotaxis, or contact response, was involved in the reverse turning reaction was unavoidable. However, it is doubtful that a simple thigmotaxis could account for quantitative compensatory turning. The 0° corridor served as a control for the thigmotactic component of reverse turning. In both experimental series the mean response to this corridor was closer to 0° than for any other forced angle. Of course, a mean response of 0° could result if the millipedes turned strongly with equal frequency to right and left. On the other hand, for the responses to the 0° corridor the variance, which would indicate the degree of strong right and left turning, was not significantly larger than the variances for other forced turns. Thus, a large thigmotactic response was not expressed on runs through the 0° corridor. Furthermore, it is presumable that thigmotaxis should influence responses for all forced turns, equally, since all such turns would tend, in the narrow corridor, to bring every animal into contact with the outside wall of the turn. It was observed that sometimes after making a forced turn the millipede would not follow the outside wall of the turn. Instead, it would remain close to the inside wall, but would constantly tap the outside wall with its antenna. Upon emergence from the corridor the animal would alternate its turning in the expected direction.

The tendency of the animal to follow by means of olfactory cues the trail of its own or another animal's earlier run did not appear to be an important factor. This is indicated, for instance, by the highly significant individual differences presented in Table II and Figure 3. Further evidence was provided by a brief preliminary experiment in which the same sheet of paper was used as the orienting surface for a series of forced turns. Although each animal had the opportunity of following its own or another millipede's trail for the same or different forced angles, the results were the same as for the experiments reported here, namely, significant differences among individuals and angles.

The effect upon reverse turning of varying the angle of the forced turn has been examined in boxelder bugs by Dingle (1964a). He measured the response to a range of forced angles by determining at the choice point the percentage of bugs that made a right-angle turn in the opposite direction rather than continuing straight ahead. He found that the percentages exhibiting reverse turning after 0°, 30°, 45°, and 60° forced turns were approximately the same, ranging from 19% to 23%, while the percentages after 75° and 90° forced turns were significantly greater, being 44% and 47%, respectively. He concluded (p. 119), "Rather than a steady increment in correcting as the amount of turn increased, there was a certain critical angle at which a marked and significant increase occurred." On this point the behavior of boxelder bugs apparently contrasts with that of the millipedes, which showed significantly different responses to all the angles used by Dingle. On the other hand, it is possible that the boxelder bug, too, possesses a capacity to respond to smaller forced angles but did not manifest this capacity under the conditions of Dingle's experiments. Two possible explanations for the apparent absence of this capacity are presented here. First, it was suggested in Series I (Fig. 3A) that millipedes did not respond to forced angles of 15° and 30° when these were presented first in sequence. Only after several forced runs through the corridor did a response to these smaller forced angles become evident. Possibly boxelder bugs require several forced runs before responding to forced angles smaller than 75°. If Dingle gave the bugs only a single trial, they may not have been in a satisfactory physiological state for evincing a response to small angles. Second, the apparent absence of correcting for small forced angles may be due to the fact that the response was measured with a dichotomous scale, in which the bug was forced to chose between the alternatives of making a 90°-reverse turn or no turn at all. Although boxelder bugs may be capable of precise compensation for forced angles between 0° and 60°, even on the first run, this gradient of response could be masked if the correcting tendency were not strong enough to produce right-angle turning, and so led to a predominant choice of the straight path. Supporting this possibility would be the fact that no critical angle was found when Dingle used a continuous scale, that is, measurement of angular turning tendency over a 180° arc, to quantify the response of bugs to various curved causeways.

It is of interest to compare the reverse turning tendency reported here with the homostrophic reflex described for millipedes by Crozier and Moore (1923). In this latter reaction a lateral displacement of the tail brings about a compensatory turning of the head so that its orientation is parallel and in the opposite direction to that of the tail. Jander (1963) has distinguished in any taxis mechanism two basic components, an afferent angle sensing and directing one and an efferent coordinating one. It may be that the two component mechanisms are similar in reverse turning and the homostrophic reflex, but the latter does not require a memory for the angle of bending of the body as does the reverse turning reaction. Possibly, however, Crozier and Moore were observing a memory for angle when they reported (1923, p. 600), "The fact that the reflex may be somewhat delayed increases the appearance of 'intelligent' pursuit of a straight path."

Precautions were taken to reduce the significance of the light source as an external orientative reference cue for menotactic, or compass, reactions. This, plus the similarity of the response to the homostrophic reflex, which is dependent upon proprioceptive cues, suggests that the reverse turning reported here is basically a kinesthetic response. Such an internally controlled kinesthetic response would, of course, nicely supplement in a functional manner any externally controlled responses, including menotaxis and astrotaxis, which serve to maintain the organism on a directed course.

It was not determined if any visual input at all was necessary for quantitative reverse turning in *Trigoniulus*. Apparently it plays a role in the boxelder bug, since blinding the bug diminishes its turning tendency (Dingle, 1964a). For a tropical millipede which normally inhabits the gloomy floor of the rain forest it is conceivable that photoreception would be relatively less important than proprioception for the response.

It should be noted, however, that the experiments as performed did not eliminate the possibility that pervasive extra-maze factors served as spatial references for a compass reaction. The recent demonstrations that animal orientation may be affected by very weak magnetic, electrostatic, and gamma radiation fields (Brown, 1962a, 1962b, 1963; Schneider, 1963) indicate that at least such a possibility should be considered.

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Summary

1. The millipede, *Trigoniulus lumbricinus,* when forced to crawl through a corridor containing an abrupt turn, tended to turn upon emergence from the corridor at an angle which was opposite and approximately equal to the angle of the forced turn.

2. The response held for forced angles ranging in size up to at least 120°. For forced turns greater than 60° the amount of reverse turning was less than the amount of forced turning. On a statistical basis the millipedes appeared to be capable of distinguishing 10° differences in the angle of the forced turn.

3. There were highly significant differences in the response patterns of individual millipedes to a graded series of forced angles.

4. The turning tendency was significantly increased following a series of turns in the same direction.

5. The precision and sensitivity of the reverse turning response suggest that it is an important orientation reaction for millipedes. A brief survey of the literature on certain maze-running phenomena, reverse turning and spontaneous

alternation, further suggests that the response is a type of kinesthetic orientation which may prove to be widespread in animals.

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THE RELATION OF SOROCARP SIZE TO PHOTOTAXIS IN THE CELLULAR SLIME MOLD, DICTYOSTELIUM PURPUREUM[1]

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One of the most striking features of the cellular slime molds is orientation towards light during the migrating and culminating phases. This has been known since the work of the early workers, and more recently we were able to show that the sensitivity of this reaction is truly remarkable, although we suggested incorrectly that the light and heat response might be explained by a similar mechanism (Bonner, Clarke, Neely and Slifkin, 1950). Gamble (1953) was the first to show that the light response was so sensitive that it was necessary to postulate a separate photoreceptor and this has been confirmed in detail by Francis (1964), who was even able to obtain a rough action spectrum of the photosensitive pigment.

For some years in our laboratory we have attempted to demonstrate phototaxis in vegetative or aggregation amoebae with no success. Samuel (1961), in particular, paid attention to this problem as has Francis (1964), and we have repeated a number of the obvious experiments during the course of this study. But in no case was any photo orientation demonstrated as it was in Davenport's (1897) early experiments on large soil amoebae.

The purpose of this study was to determine whether or not small cell masses, which would be intermediate in size between single cells and the usual large pseudoplasmodia, would respond to undirectional light with the same effectiveness as the larger masses, or whether they would show intermediate responses. As will be seen, for a given light intensity, the effectiveness of the response definitely decreases with size. The phototactic response is correlated with sorocarp size.

METHODS

The majority of these experiments were done on *Dictyostelium purpureum* (strain No. 2), although a few comparative studies were made with *Polysphondylium violaceum* (strain No. 6), *P. pallidum* (strain No. 4), *Acytostelium leptosomum,* and *Protostelium mycophaga.* In all cases they were grown on *Escherichia coli* which had been painted on the surface of 2% non-nutrient agar.

When aggregation was largely completed and well formed centers were evident, these were punched out with a small glass tube and placed on the surface of a large block of agar within a lucite box 3 × 3 × 9 cm. in size (Fig. 1). These were in turn put in a darkroom so that each individual center resided at the same given distance from the light source. There was no temperature regulation of the dark

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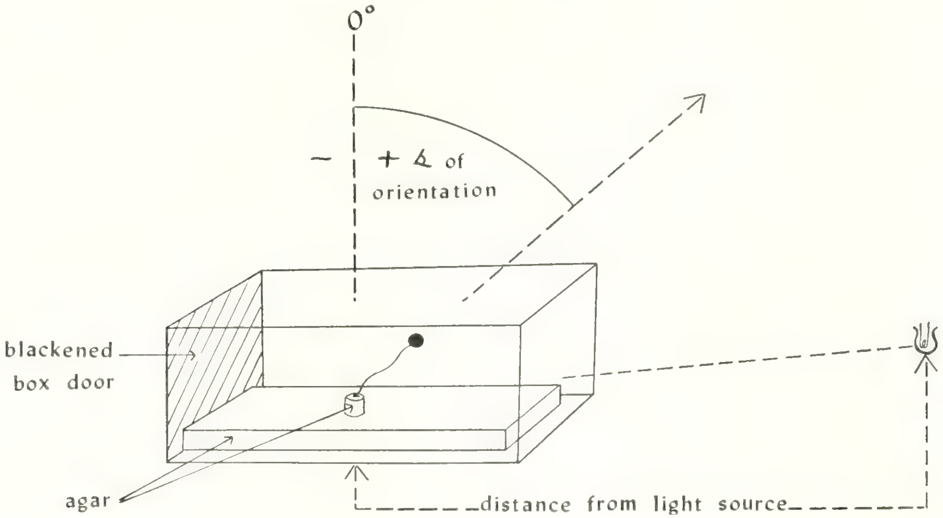

FIGURE 1.

room and while the temperature was relatively steady during any one experiment, the temperature at which the experiments were run varied from 24° C. to 27° C.

The light source was a 6.5-volt G.E. headlight lamp No. 1493. It was set with a transformer so that it received 1.14 amps. and 0.90 volts. The intensity of the light was checked before and after an experiment with a photocell and a galvanometer.

After the pseudoplasmodia had fruited, the plastic boxes were placed upon their sides under a dissecting microscope and camera lucida drawings were made of the sorocarps. The angles were determined by drawing a line from the base through the upper end of the stalk at the point where it enters the sorus (Fig. 1). The vertical position was considered 0°, and any deviation towards light was given a positive angle value, while orientation away from the light was given a negative value.

TABLE I

Mean angles of deviation from the perpendicular for different size sorocarps at three different distances from a light source. The standard deviations are given for each mean and the number of cases in parentheses (These are plotted in Figure 2)

Height of sorocarp in mm. (grouped in size classes of ±0.2 mm.)	Angle of deviation from the perpendicular with the light at different distances		
	1 foot	6 feet	18 feet
0.2	10° ± 23°(9)	3° ± 20°(7)	1° ± 19°(10)
0.6	10° ± 30°(14)	−4° ± 10°(8)	7° ± 28°(12)
1.0	42° ± 26°(14)	3° ± 14°(14)	−3° ± 14°(16)
1.4	37° ± 31°(13)	21° ± 28°(12)	2° ± 22°(22)
1.8	48° ± 36°(10)	33° ± 17°(9)	−4° ± 14°(22)
2.2	53° ± 15°(5)	38° ± 23°(6)	8° ± 20°(20)
2.6	52° ± 18°(6)	35° ± 19°(9)	2° ± 50°(15)

RESULTS

As can be seen from Table I and Figure 2, the experiment was run at three different distances, 1, 6, and 18 feet. Although the standard deviations show considerable variability in the response, it is clear that at one foot the larger sorocarps responded while the smaller ones did not. At 18 feet there was no response, even of the largest sorocarp. In fact this agrees with a control run in the total darkness, involving 60 sorocarps. At 6 feet there was an intermediate response. For instance if one looks at the sorocarps that are 1 mm. long, then only those at 1 foot are oriented while those at 6 and 18 feet are not. However, at 1.4-mm. the ones at 6 feet from the light source are definitely oriented, although the

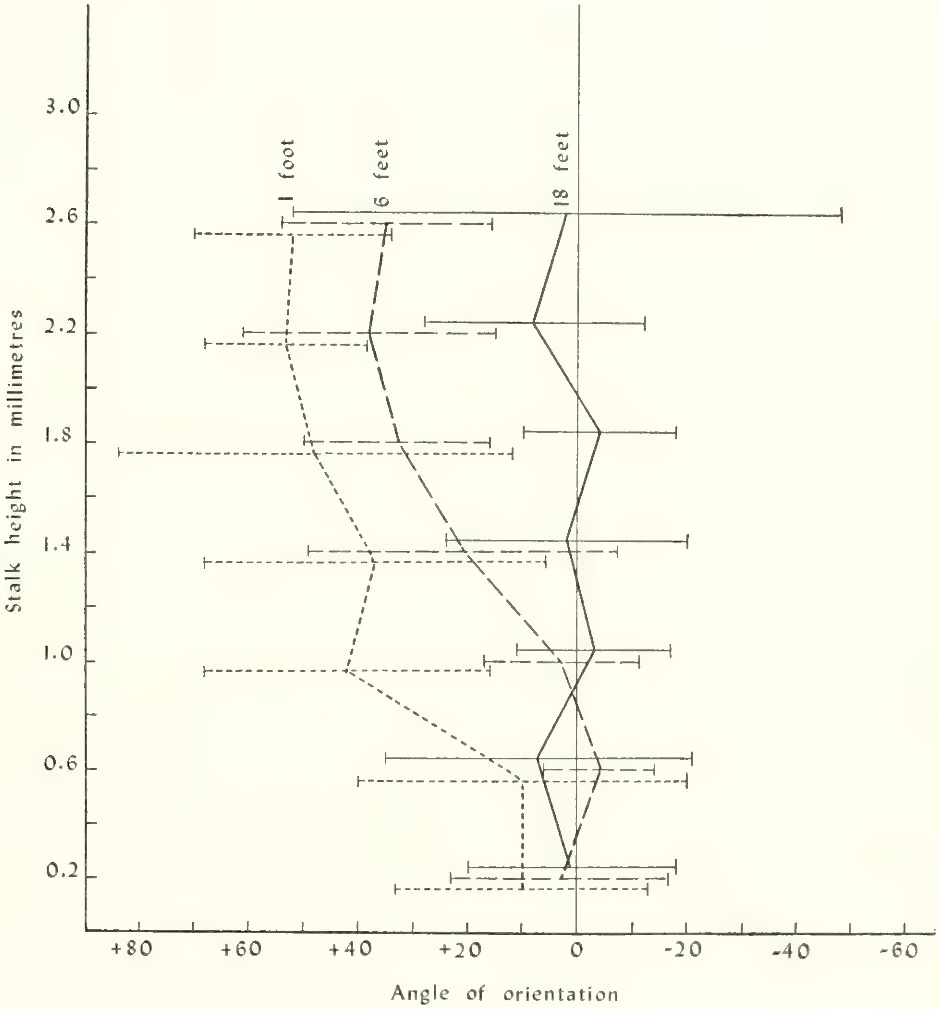

FIGURE 2.

angle of orientation is less than those at one foot. It should be added that this same result was obtained in a preliminary experiment involving 59 cases in which the intensity of the light was changed rather than changing the distance. This experiment, however, has the serious objection that the spectrum of the tungsten filament changes with intensity and therefore the data have not been included here.

These results were repeated in a brief preliminary experiment comparing five species. In *Protostelium*, which has no aggregative phase and consists of a single cell raised on a minute stalk (Olive and Stoianovitch, 1960), and in *Acytostelium*, which is a very small species with an acellular stalk (Raper and Quinlan, 1958), there was no orientation at all, even with strong light intensities. The remaining three were tested at the same light intensity and *Polysphondylium pallidum* (21 cases) was the least sensitive, *Dictyostelium purpureum* (37 cases) next, and *Polysphondylium violaceum* (18 cases) the most sensitive and showed the lowest threshold. It is of interest that only the main stalk in *Polysphondylium* showed orientation; the small side whorls never leaned towards the light, which might be expected on the basis of their small size.

Table II

Mean angles of deviation from the perpendicular for different-size sorocarps of Polysphondylium pallidum. In the first column of angles two sorocarps are repelling each other, and in the second column a single sorocarp is orienting away from a vertical wall of agar. In both instances the distance between the sorocarp base and the other sorocarp or agar varies from 0 to .53 mm. The number of cases is indicated in parentheses

Height of sorocarp in mm. (Grouped in size classes of ±.1 mm.)	Angle of deviation from perpendicular	
	Two sorocarps	Sorocarp and agar wall
.2	35°(1)	7°(1)
.4	31°(8)	16°(2)
.6	19°(12)	5°(5)
.8	20°(8)	13°(9)
1.0	9°(2)	8°(5)
1.2	20°(1)	17°(3)

During the course of these experiments the question arose as to whether light might directly affect the rate of movement within the cell mass. This was tested by subjecting migrating masses of *Dictyostelium discoideum* to alternating periods of light and dark (with the microscope lamp in the darkroom) and measuring the rate of movements. In 18 separate experiments, most of them run for at least four hours, the light was alternately turned on and off at hourly intervals. The overall average rate during the dark periods is almost identical to that during the light period (Light = 1.6 mm./hr.; Dark = 1.7 mm./hr.) and if one scores whether light has produced an increase or decrease in speed (or conversely the dark), both occur with equal frequency. A few further experiments were run with the light and dark alternating at 10-minute intervals and again there was no difference in the rate. These results are consistent with those of Francis (1964), who showed amoebae separated from a slug and subjected to strong increases in light intensity did not exhibit any change in speed.

Besides the effect of light it should be recorded that two other types of orientation were tested.

By putting the plastic boxes on end in the total darkness it was possible to test the effect of gravity. The results do not show, as might be expected, that the large sorocarps droop. In 54 cases there is no clear downward trend, but the variance appears greater than the right-side-up controls in the dark and the right-side-up sorocarps 18 feet distant from light (Fig. 2). It would be interesting to know if the mechanism which controls orientation in the dark has difficulties in operating with precision when the sorocarp arises on a vertical wall.

Presumably the method of orientation in the dark is entirely by gas gradients (Bonner and Dodd, 1962a). The old data from those experiments were reexamined and it is possible to compare the amount of repulsion between sorocarps and the size of the sorocarp. It is obvious from the results shown in Table II that the small sorocarps are as effective in orienting to gas gradients as large ones, and that this is true for a number of species.

DISCUSSION

The discussion of these results will be divided into two sections: the mechanism of orientation, and the question of adaptation.

Unfortunately, these experiments tell us very little about the mechanism of orientation, but then perhaps this is not too surprising because despite all the detailed work on phototropism in *Phycomyces,* it is still far from understood. Orientation in the slime molds is similar to *Phycomyces* in that Francis (1964) showed that a small beam of light hitting one side of slug will cause the slug to bend away from that side, thus showing a parallel to Buder's (1920) experiment in *Phycomyces.* During the course of our work we repeated another experiment of Buder and placed the slime molds in mineral oil. As in *Phycomyces* they oriented away from the light and all of this substantiates Francis' (1964) conclusion that the lens effect is operative in the slime molds.

Unlike *Phycomyces,* there is no evidence that light affects the speed of movement, even for brief periods. From this Francis (1964) makes the reasonable suggestion that perhaps the light might be affecting the extensibility of the slime sheath which in turn directs the movement.

Another difference is that in *Phycomyces* the smaller sporangiophores are more sensitive to a given unilateral light than large ones, as Castle (1964) has shown. This is exactly contrary to the results here. From this we must conclude that the limiting factors are different for the two systems, but what they might be for the slime molds is difficult to surmise. It is not just light intensity, for if the lens effect operates, it should if anything be more effective in the small fruiting bodies at a given light intensity. Therefore, there must also be some limitation within the sorocarp itself. Something within the cell mass is quantitatively below threshold, but the threshold can be raised to some extent if the undirectional light intensity is raised.

It is interesting that this should be in marked contrast to the orienting effects of gas gradients. Here the smallest sorocarps are as responsive as the large ones. The fact that single amoebae can also orient very effectively in chemical gradients, such as the acrasin gradient, food gradients (Samuel, 1961) and mutual repulsion gradients (Samuel, 1961), is perhaps consistent with the notion that there is no size threshold for these chemical effects.

To turn now to the question of adaptation, if orientation to light has adaptive value, then clearly there will be a selection pressure for large slime molds. Increase in size, however, may result in other features which are inadaptive, and some sort of balance must be reached.

Size is to some degree fixed for a particular species or a particular strain and this is in part due to the spacing mechanism (Bonner and Dodd, 1962b; Bonner and Hoffman, 1963) and in part due to another mechanism which operates at higher amoeba densities (Hohl and Raper, 1964). Since there are different-sized species in nature we must presume that there are a number of adaptive advantages and disadvantages in size. This same question has been examined for different-sized *Hydra* by Slobodkin (1964), where he considers the whole question in terms of strategy, balancing the advantages and disadvantages in different ways. Here we can say that the ability to orient towards light improves with increased size, and if for any one species, in a particular environment, this is advantageous, there will be selection for size increase.

The authors are indebted to Dr. L. S. Olive for cultures, and to Dr. D. W. Francis for his critical reading of the manuscript.

SUMMARY

Large fruiting bodies of the cellular slime mold, *Dictyostelium purpureum*, orient more effectively towards a source of light of low intensity than do small ones. The threshold of sensitivity can be changed either by changes in size of the sorocarp or by changes in the light intensity. However, in chemical gradients small cell masses are as sensitive as large ones. Therefore, if orientation to light is of adaptive value, selection pressure for size increase would be expected.

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VARIABILITY IN LARVAL STAGES OF THE BLUE CRAB, CALLINECTES SAPIDUS[1]

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Renewed interest in crustacean development, coupled with techniques which permit the rearing of larvae representing a variety of groups, has resulted in an increase in descriptions of larval stages and comparisons of similar stages obtained from laboratory rearing and from the natural environment. Comparisons of laboratory-reared larvae with those from the plankton frequently include observations on certain variations, either in morphological characters within stages thought to be comparable or variations in the total number of larval stages. Some variability has been noted in larvae representing many of the groups within the Crustacea. Larvae of the Brachyura, however, while receiving perhaps greater attention than larvae of other closely related groups, have not been found generally to exhibit variability in either the number of stages or in morphological details within any one particular stage. The two exceptions are larvae of the blue crab, *Callinectes sapidus* (Costlow and Bookhout, 1959) and larvae of the stone crab, *Menippe mercenaria* (Porter, 1960). In studies on larvae of these two species of crabs, an "extra" stage was described, intermediate between the zoeal stage generally considered to be the final zoeal stage, and the megalops stage.

The present study was undertaken to determine if variability in larvae of *Callinectes sapidus* reared under controlled laboratory conditions was limited to the occasional occurrence of an "extra" stage late in development or if irregularities occurred in the molting pattern of earlier larval stages which could result in morphological differences which might be interpreted as separate or intermediate zoeal stages.

The author is indebted to Miss Judith Payne and Mr. Charles Lynch for their assistance throughout the study.

METHODS

The larvae of the blue crab, *Callinectes sapidus* Rathbun, were obtained by removing the eggs from the pleopods of four female crabs and hatching them in compartmented boxes maintained on an Eberbach variable speed shaker (Costlow and Bookhout, 1960). At the time of hatching 80 first stage zoeae were segregated, into Syracuse watch-glasses, one zoea per watch glass, and maintained in a culture cabinet at 25° C. The larvae were changed each day to freshly filtered sea water,

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[1] These studies were aided by a contract 14-17 0002-60 between the Bureau of Commercial Fisheries and Duke University.

30 p.p.t., and fertilized *Arbacia* eggs and recently hatched *Artemia* nauplii were added. Before the larvae were changed the watch glasses were examined under a dissecting microscope. If molting had occurred, both the shed exoskeleton and the zoea were examined under a compound microscope to establish what morphological changes had occurred. For purposes of comparison, the descriptions and figures of the seven zoeal stages of *C. sapidus* (Costlow and Bookhout, 1959) were used as a basis for differentiating between the sequence of zoeal stages. Any variability in appendages or abdominal segments was recorded throughout progressive molts.

RESULTS

During larval stages I–IV, the morphological characteristics previously described by Costlow and Bookhout (1959) were found to be consistent. Within some series of *C. sapidus* larvae, however, variability in molting was observed after stage IV. The variability can be grouped into three general categories: (1) a molt without any perceptible morphological changes; (2) a molt which eliminated or "skipped" one of the previously described larval stages; and (3) a molt which resulted in a larva with a combination of morphological characteristics previously described for two separate larval stages.

Table I represents the stages of zoeae observed at the time of each molt for one series of 20 *C. sapidus* larvae segregated as stage IV zoeae. Forty-five per cent

TABLE I

Stage of larval development at the time of each molt for 20 zoeae of Callinectes sapidus isolated as stage IV larvae. Roman numerals indicate stage of development

Zoea Number	Molt number					
	3	4	5	6	7	8
1	IV	V	VI	VII	Meg	
2	IV	VI	VIII	Meg		
3	IV	V	V–VI	VI–VII	VIII	Meg
4	IV	V	VII	Meg		
5	IV	V	VI	VII	Meg	
6	IV	V	V–VI	VII	VII–VIII	Meg
7	IV	V	VI	VII	Meg	
8	IV	V	VI–VII	Meg		
9	IV	V	VI (dead)			
10	IV	V	V–VI	VI–VII	Meg	
11	IV	V	VI	VII	Meg	
12	IV	V	VI	VII	Meg	
13	IV	V	V–VI	V–VI	VII	Meg
14	IV	V	VI	VII	Meg	
15	IV	V	VI	VII	Meg	
16	IV	V	VI	VII–VIII	Meg	
17	IV	V	VI	VII	Meg	
18	IV	V	VI	VII	Meg	
19	IV	V	VII	Meg		
20	IV	V	VI	VII	Meg	

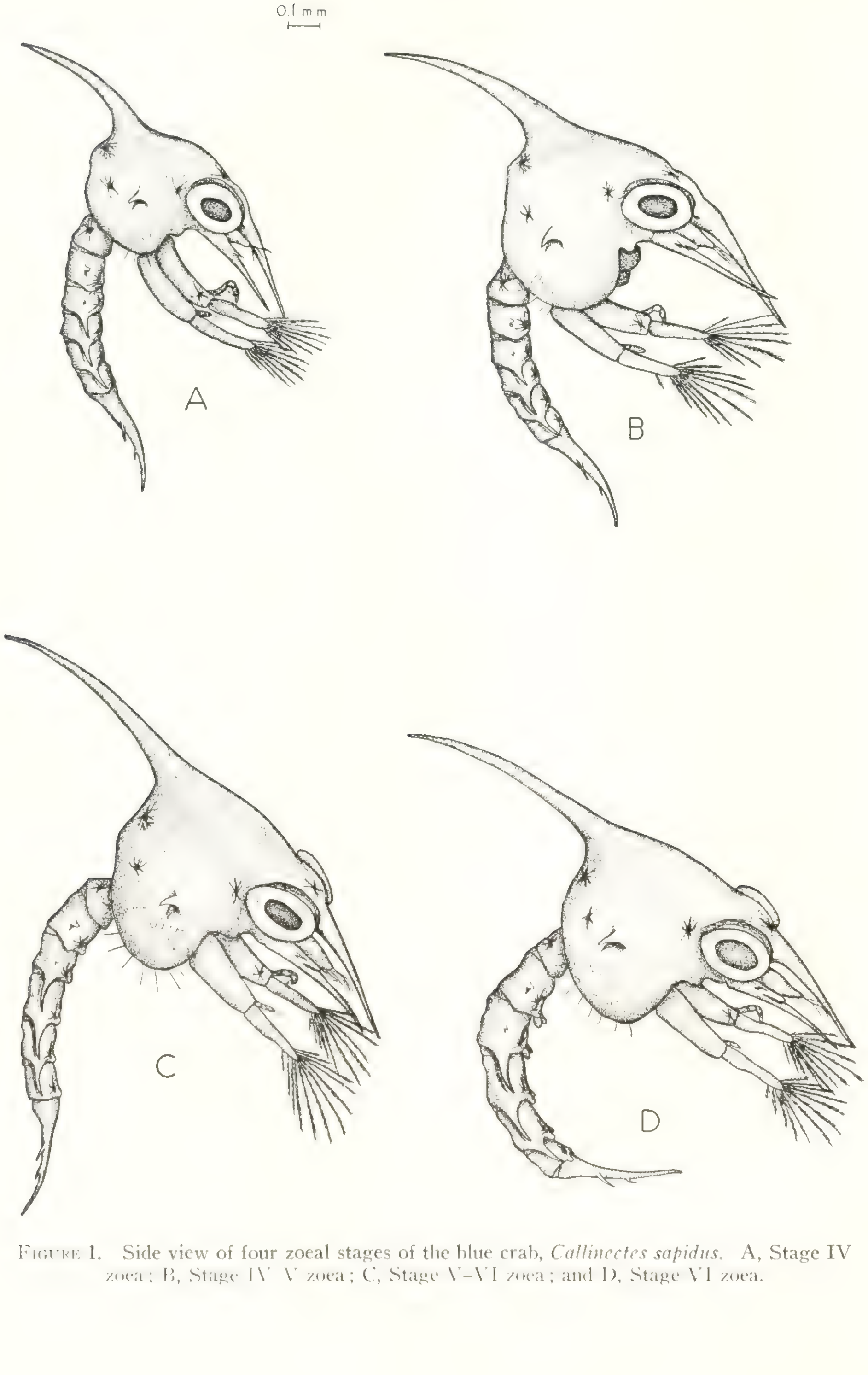

FIGURE 1. Side view of four zoeal stages of the blue crab, *Callinectes sapidus*. A, Stage IV zoea; B, Stage IV–V zoea; C, Stage V–VI zoea; and D, Stage VI zoea.

of these larvae exhibited some variability during subsequent molting. In the three series which were studied, variability was observed in from 40% to 63% of the larvae.

As shown in Table I, the frequency of molts without any perceptible morphological change was low (Zoea #13). The few larvae which were observed to molt without any morphological change usually exhibited the morphological characteristics previously described for the next stage (Costlow and Bookhout, 1959) at the time of the next ecdysis and proceeded to molt normally in remaining larval molts. The complete elimination of one of the larval stages by "skipping" occurred more frequently (Table I, Nos. 2, 4, 19). In rare instances this type of variability was observed over several consecutive molting periods for the same zoea. The third type of variability, a molt resulting in a larva which combined morphological characteristics from two stages, was the most common (Table I, Nos. 3, 6, 8, 10, 13, 16). The "combined stages" most frequently observed were stage "V–VI" and stage "VI–VII." The morphological characteristics which usually were combined in these two stages were the antennae and maxillipeds of stage VI combined with the abdominal segments of stage V (Fig. 1, B), or the antennae and maxillipeds of stage VII combined with the abdominal segments of stage VI (Fig. 1, C). It should be noted that in a "combined stage" the more advanced characteristics were invariably in the anterior portion of the zoea; *i.e.,* in a stage "VI–VII" zoea the anterior portion resembled the stage VII larva, as described by Costlow and Bookhout (1959), while the abdominal segments and development of pleopod buds were those previously figured for a stage VI zoea. A greater range of variability between stages was not observed.

In many instances "combined stages" did, at the next molt, display the characteristics described for a single stage, but some larvae continued to molt with more advanced anterior development until the megalops stage was reached (Table I).

The variability which was observed was accompanied by differences in the actual number of larval stages or molts required for development to the megalops stage. Some larvae (Table I), by eliminating stages V or VII, attained the megalops stage in six zoeal stages. Other larvae, which did not eliminate specific stages, required seven or eight zoeal stages to complete development to the megalops stage.

DISCUSSION

Variability has been observed within the developmental stages of Crustacea representing a number of specific groups. Within the Cirripedia there has been general agreement on the occurrence of 6 naupliar stages prior to the cyprid stage. Two authors have described a seventh naupliar stage in rearing studies on *Balanus amphitrite albicostatus* (Ishida and Yasugi, 1937) and *Balanus amphitrite hawaiiensis* (Hudinaga and Kasahara, 1941). In both instances, however, the only difference between the sixth and seventh naupliar stages was the presence of paired eyes, a feature which is known to develop late in the sixth naupliar stage of all other species of barnacles described to date. It should be noted that while the two studies mentioned dealt with laboratory-reared material, differences have also been reported in barnacle nauplii obtained from the plankton. Norris and Crisp (1953) described "developmental intermediates" in nauplii of *Balanus perforatus,* and Jones and Crisp (1954) found minor differences in setation of nauplii of *Balanus improvisus*.

Within the euphausids, Fraser (1936) described some "skipping" in the number of stages of reared larvae of *Euphausia superba*. In studies on larvae of *Nyctiphanes simplex* obtained from the plankton, Boden (1951) described six "dominant" furcilia stages but suggested that as many as 19 may occur in development.

Larvae of several species within the Natantia have also been shown to vary in number of developmental stages or in minor morphological features within any one specific stage. Renfro and Cook (1963) described minor differences in the setation of naupliar appendages of the penaeid shrimp, *Xiphopeneus kroyeri*, reared in the laboratory. Broad (1957a), rearing the larvae of *Palaemonetes pugio* and *P. vulgaris*, found skipping of stages and commented that the rate of development of the larvae and the frequency of molting were independent.

Within the Reptantia, studies on the planktonic phyllosoma larvae of the spiny lobster, *Panulirus argus* (Lewis, 1951), disclosed considerable variation between individuals in the same stage during the later developmental stages.

Accounts of variability among larvae of the Anomura are available from rearing studies, as well as from studies on larvae obtained from the plankton. Working with reared larvae, variability was described for *Pleuroncodes planipes* (Boyd and Johnson, 1963), *Calcinus tibicen* (Provenzano, 1962a), *Coenobita clypeatus* (Provenzano, 1962b) and *Emerita talpoida* (Rees, 1959), while Johnson and Lewis (1942) describe variability in larval development of *Emerita analoga* obtained from the plankton.

Within the Brachyura, or true crabs, the references to variability in larval development are limited to the description of an "extra" stage, late in the zoeal development of *Callinectes sapidus* (Costlow and Bookhout, 1959) and *Menippe mercenaria* (Porter, 1960) reared in the laboratory. A zoea, comparable to the "extra" eighth zoeal stage of *C. sapidus*, has also been found in the plankton (Nichols and Keney, 1963).

The foregoing resumé of variability in larvae of widely varying groups of Crustacea, while not intended to be complete, serves to illustrate that variability does exist in larvae which develop through a series of successive stages by molting, be they reared larvae or larvae obtained from the natural environment. A number of factors have been investigated as possible causes of variability in crustacean larvae. Several authors have noted the occurrence of a "pre-zoea" stage in the development of the blue crab, *C. sapidus* (Robertson, 1938; Churchill, 1942; Sandoz and Rogers, 1944; Costlow and Bookhout, 1959). The "pre-zoea" occurs most frequently when the eggs are hatched in a salinity lower than 20 p.p.t. but this example of variability early in larval development is more closely related to abnormal hatching than to variations in rate of larval development or an actual stage of development. Other studies on the effect of salinity and temperature on larval development have either noted that these two environmental factors did not affect the number of larval stages (Costlow, Bookhout and Monroe, 1960; 1962) or the authors did not comment on any variability which may have existed (Coffin, 1958; Templeman, 1936a).

A limited number of studies on the possible effects of light, or photoperiod, on larval development have failed to show any positive relationship (Templeman, 1936a; Costlow and Bookhout, 1962), although earlier work by Huntsman (1924) suggested that strong sunlight did reduce the per cent survival of young larvae of *Homarus americanus.*

A third factor, diet, has been suggested as the cause of variability in larval development of several species of Crustacea. Templeman (1936b) attributed an occasional "extra" stage in the development of *Homarus americanus* to a reduction in available food. Broad (1957b) found that the rate of development of *P. pugio* and *P. vulgaris* larvae, as well as the number of larval stages, was associated with the amount of available food as well as the nature of the diet. Chamberlain (1961), working with the larvae of crabs belonging to the Xanthidae, found that although different diets affected the rate of development as well as the survival, the number of zoeal stages was not changed. In studies on the larvae of *C. sapidus,* the diet was adequate in quantity and has, during the past five years, contributed to the successful and normal development of many thousands of zoeae of 30 species of crabs, including the zoeae of *C. sapidus.* The abundance of food, however, does not necessarily indicate that it is ingested or effectively utilized by all larvae.

Previous studies on the larval development of other species of crabs have emphasized the regularity in molting frequency of the zoeae when environmental factors are optimal (Costlow, Bookhout and Monroe, 1960; 1962). While the mechanisms which control the regular sequence of molts in larval forms are not known, suggestions have been made on the basis of recent studies (Costlow, 1961, 1963a, 1963b). Earlier work by a number of investigators (Orlamunder, 1942; Pyle, 1943; Dahl, 1957), as well as the recent study by Hubschman (1963), described groups of cells in developmental stages of a number of Crustacea which are similar to the X-organ of the adult eyestalks. The stage of development when these cells first become functional, producing the molt-inhibiting hormone which, with the molt accelerating-hormone of the Y-organ, regulates the cyclic molting pattern of the adult crab, is not known for any larval form. Costlow (1963a), working with megalops of *C. sapidus,* has suggested that early in larval development only the accelerating hormone of the Y-organ is present. Late in larval development, perhaps at the beginning of the megalops stage, the X-organ sinus gland complex of the eyestalk becomes functional, prolonging the intermolt period of the megalops and eliminating the established frequency of molting of the zoeae. Results of studies on the effect of eyestalk extirpation on metamorphosis, molting frequency, and growth of *C. sapidus* megalops definitely established that molting and growth were independent and apparently controlled by separate mechanisms (Costlow, 1963a). If, as suggested by a study of molting and regeneration of chela in megalops of *C. sapidus* (Costlow, 1963b), molting has priority over regeneration, the regular pattern of molting which appears to be superimposed on the development of larval morphological features may also have priority over the normal pattern of morphological development. Under these circumstances one might expect that any factor which altered the rate of morphological development, but did not affect the mechanisms which controlled molting, would be observed as "skipping" of larval stages or "extra" larval stages.

The three general types of variability which were observed in the present study of *C. sapidus* larvae could serve as examples. In the first type, molting without any perceptible morphological changes, the frequency of molting was maintained while the mechanisms responsible for continued development were completely inhibited. In the second type of variability, the elimination or "skipping" of a larval stage, the molting frequency was again constant while the mechanisms controlling development were accelerated. The third type of variability was a molt which resulted in a larval stage which combined morphological characters normally attributed to two zoeal stages. The molting pattern, still constant, would appear to have been preceded by normal or accelerated development in the anterior portion of the larvae, while in the posterior portion, development was inhibited or normal.

It should be noted that in virtually all accounts of variability in number of larval stages, "skipping" or "extra" stages first become apparent late in larval development. The two cases described for the Brachyura involved a zoeal stage beyond the stage which is normally considered the final zoeal stage. Morphologically the "extra" zoeal stage had certain features, usually in the anterior portion of the animal, which closely resembled those features described for the megalops stage. In the present study, variability in larval stages was never observed before the fifth zoeal stage. As the majority of the larvae of *C. sapidus* pass through seven zoeal stages, the fifth stage may be considered "late" in development. If this variability is caused by the malfunction of an endocrine mechanism, the fact that the variability is limited to the later stages may be due to a gradual decline in secretory activity of the endocrine mechanisms rather than an abrupt cessation of activity. Another possible explanation should also be considered. Within the Brachyura the number of zoeal stages within any one species ranges from two (*Libinia,* unpublished) to seven (*Callinectes:* Costlow and Bookhout, 1959), with a number of species having either 3, 4, or 5. Only within the Portunidae, the family to which *Callinectes* belongs, has the number of zoeal stages been shown to exceed five, and this family is considered to be the most primitive group within the Brachyura (Lebour, 1928). Thus, it is possible that this variability, so extremely rare in the larvae of other species of Brachyura with a relatively small number of zoeal stages, is associated with the primitive nature of the Portunidae.

Experiments in our laboratory have shown that "extra" or supernumerary zoeal stages could be induced in the development of other species of crab larvae by the removal of both eyestalks prior to a critical period in larval development (Costlow, 1963c). Variability in these experimental larvae, similar in general external appearance to those described for extra stages of *C. sapidus* (Costlow and Bookhout, 1959) and *M. mercenaria* (Porter, 1960), occurred late in larval development and were directly associated with the absence of, or the reduced titer of, some substance which is elaborated within the neurosecretory centers of the larval eyestalks. If the Y-organ is present and functional in larval stages, activating the regular sequence of molts through the secretion of the molt-accelerating hormone without the inhibiting effect of the X-organ, removal of the eyestalks during the zoeal stages should not alter the molting frequency of the zoeae. Recent experiments (unpublished) would indicate that this is the case and further substantiate the concept that the pattern of development of morphological features is independent of molting and controlled by a separate mechanism within the eyestalks.

In the sequence of development of normal zoeae, *i.e.,* zoeae which have not had eyestalks removed, variability may also be linked to malfunction of endocrine systems which do not permit the development of morphological features in the pattern generally regarded as "normal," to keep pace with the frequency of larval molting which is controlled by separate endocrine systems. Under this dual system of control, insufficient food, dietary deficiencies, or the absence of certain organic or inorganic trace elements in sea water could prevent or delay the normal functioning of the endocrine mechanisms which control development. Externally these deficiencies, as well as the resultant malfunctioning of the endocrine system, would be manifest as variability in the number of larval stages or in minor differences in morphological characters of the larvae.

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ON THE REPRODUCTION AND LARVAL DEVELOPMENT OF STREBLOSPIO BENEDICTI WEBSTER[1, 2]

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Streblospio benedicti Webster is a common but inconspicuous polychaete which abounds in many habitats along the eastern United States seaboard from New England to North Carolina. It is often present in estuaries, on beaches, or where the substratum consists of fine mud with considerable debris (Hartman, 1945).

This worm is very unusual for it is larviparous. The only published accounts of the reproduction or larval development of this species are brief comments by Campbell (1957) and Jones (1961).

The investigations reported here include descriptions of the development of larval specimens reared from plankton isolates and of larvae released by adults maintained in the laboratory.

METHODS

During June and July of 1963, *Streblospio* adults were collected from the Pequotsepos section of the Mystic River Estuary, where an abundant population inhabited a substratum of fine mud. In the laboratory the worms were maintained in fingerbowls containing sediment. The sediment came from the same area as the worms but was screened free of macrofauna, washed in distilled water, and air-dried prior to use.

Pelagic larvae were obtained from two sources: (a) isolates of qualitative plankton tows taken in the Mystic River Estuary with a No. 10 net during June to October, 1962 and 1963; (b) broods released by adults maintained in the laboratory. Larvae were reared in funnels, with and without sediment, as described previously (Dean and Hatfield, 1963a).

To maintain temperature control, both funnels and fingerbowls were partially immersed in a bath of flowing water from the laboratory's salt water system. Water in all containers was changed three times a week. Sea water used for cultures was Millipore-filtered (pads of 47 μ porosity) and stored in stoppered flasks in a bath of running sea water. Both adults and larvae were fed a suspension of liver powder weekly (Howie, 1958). Larvae and adults were maintained easily under these conditions in the laboratory. Adults formed new tubes rapidly in the sediment.

For examination, larvae were placed on microscope slides with small pieces of Saran Wrap used as coverslips (Dean and Hatfield, 1963a, 1963b). This was

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effective in quieting larvae without injury. Photographs, taken through a phase microscope, were obtained with a Polaroid camera using ten-second film. A series of photographs, descriptions and sketches of characteristic parts served as the basis for the composite outline drawings.

I. Development Within the Adult

Worms were examined periodically for the presence of eggs.

Females could be distinguished from males only if the worms contained eggs or larvae. The blue-green eggs and larvae can be seen through the body wall. Uncleaved eggs measure 53 to 76 μ in diameter. The sperm of males imparts a faint milky appearance to the posterior region of the worm. This coloration is so slight that it was not observed until several dozen worms had been examined. Sperm length ranges from 100 to 130 μ. An unusually long midpiece (*ca.* 60 μ) is found in these sperm.

Thirty gravid females, ranging from 40 to 50 segments in length, were isolated in stender dishes containing a small amount of sediment. These worms were examined frequently in order to study the development of young.

All larval development was restricted to the middle portion of the worms. The first egg-containing segment ranged from the 11th to the 16th (average 12.8). The 31st segment was the last to contain larvae. The posterior 6 to 10 segments of this reproductive zone developed paired dorso-lateral swellings—the brood pouches—from which the matured larvae were released. One pair of eggs or larvae usually occupied a segment, although one pair of larvae per pouch was observed in a few instances.

The young developed in cycles, with several maturing at about the same time. The release of larvae began at the most anterior pouch and progressed segment by segment. Normally it took two to three hours for all larvae in the pouches to be liberated. Two to three days following the release of a larval brood, another set of larvae occupied the brood pouches. These were in turn released about four to five days later. Refilling the brood pouches was sometimes associated with the disappearance of eggs from the anterior segments. However, neither eggs nor larvae were observed moving from one segment to another.

Forward of the brood pouches, development progresses in an anterior to posterior direction. Uncleaved or cleaved eggs occur anteriorly while trochophores slowly revolve in the segments near the brood pouches. When the brood pouches are refilled, the specimens occupying the posterior pouches are further advanced in development than those in the anterior pouches. Since all larvae are approximately equal in development at the time of release, either the larvae in the posterior pouches have a retarded rate of development or those in the anterior pouches have an accelerated rate.

Specimens ranging from uncleaved eggs to well-formed setigerous larvae were removed from females and examined under the phase microscope. Cleavage is spiral and leads to a trochophore (Fig. 1). The latter has a complete prototroch, one pair of eyes, sensory bristles on the head, and a neurotroch leading from the mouth. No apical tuft was observed. As development proceeds, a second pair of eyes is added, and the first segment—which is as broad as the head—becomes delimited. A distinct groove (Fig. 2) appears between the head and first segment, only to disappear later as the first segment becomes smaller than the head. A narrow, non-segmented body region extends posteriorly from segment 1. At this stage, although setae are absent, the total length approximates that of a 7-setiger larva. A mid-dorsal separation of the prototroch occurs, a telotroch develops, and within a period of about 24 hours, rapid setation and segmentation result in the formation of a 7-setiger larva (Fig. 3). As this takes place, the segments become delimited in succession from anterior to posterior, with the swimming setae being formed prior to the boundaries of a segment. No distinction can be made between noto- and neurosetae at this time, except a pair of hooded crotchets developing internally within the 7th setiger.

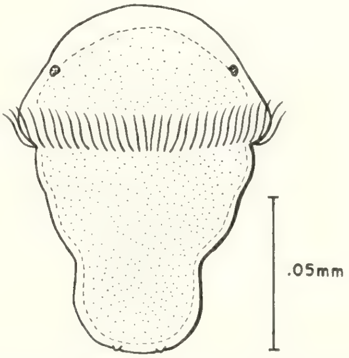

FIGURE 1. Early trochophore in dorsal view. Specimens of this stage are usually located in the female anterior of the brood pouches. Figures 2, 4 and 7 are drawn to the same scale.

All future segments are formed from a region just anterior to the pygidium. Formation of the 8th and 9th setigers takes about one day each. As setiger 9 develops, noto- and neurosetae become distinct. All notosetae are capillary, as are the neurosetae on setigers 1 through 6. Posterior to setiger 6, hooded crotchets develop and are accompanied by a stout, short, curved, capillary-tipped seta, one per neuropodium.

Larvae up to the 9-setiger stage were observed within females. Once in the brood pouches, the larvae may be released if the female is stimulated. In the laboratory, rough handling or subjection to strong light (for example, the light of a dissecting microscope lamp) may evoke the release of larvae from brood pouches. With careful handling of females, larvae were not released prior to the 9-setiger stage.

II. DEVELOPMENT OF PLANKTONIC LARVAE

1. *General*

Although thousands of *S. benedicti* larvae were observed in plankton samples taken during two summers, none were found younger than 7-setiger or older than 13-setiger stages. Seven-setiger larvae were extremely rare; the vast majority of planktonic larvae were 9- and 10-setiger; 11-setiger stages were found less frequently; and 12- and 13-setiger stages were rarely encountered. The robust anterior end and the lack of chromatophores are the most obvious features that distinguish larvae of this species from all other planktonic larvae occurring in this area. In the youngest planktonic larvae the head region is approximately twice the width of the segmented portion. In later stages this characteristic becomes less marked although there is still an illusion of greater mass in the anterior region caused by the development of palps, branchiae, and the collar of setiger 2.

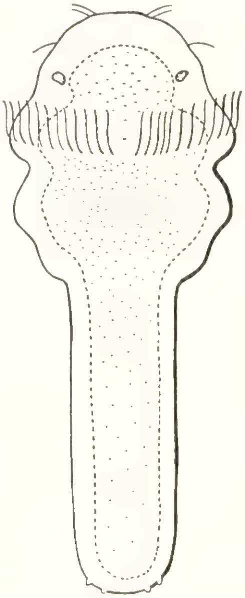

FIGURE 2. Late trochophore liberated from a brood pouch following stimulation of a female. Dorsal view.

2. *Detailed description*

All pelagic stages have four red eyes in a transverse row and neuropodial hooded tridentate crotchets beginning on setiger 7 (Figs. 3 to 7). In the 7- to 9-setiger stages, the neuropodia have a single crotchet accompanied by a short, curved, capillary seta. A second crotchet is added at the 10- or 11-setiger stage. The first six neuropodia and all notopodia have capillary setae only. When new segments are added, the crotchets are the first setae to appear. Swimming setae of early planktonic stages are very long; those of setiger 1 extend beyond the pygidium. These finely serrated setae break off at the slightest provocation and are of little diagnostic value. Eleven-setiger stages obtained from plankton tows usually lack swimming setae.

The prototroch persists through the 13-setiger stage. It encircles the swollen head region except in the dorsal midline and is outlined by a red-brown line at the base of the cilia. The prototroch is lost between the 13- and 14-setiger stage. Ventrolaterally the prototroch is borne on lobes (Fig. 4). The latter are fused anteriorly and bound the mouth laterally. A V-shaped neurotroch passes posteriorly from the triangular-shaped mouth to a ciliated pit on the anterior aspect of setiger 2 (Fig. 6).

The telotroch is present on 7- to 13-setiger stages. It is composed of 5 patches of cilia (Figs. 4, 6 and 7). The mid-ventral patch marks the anterior boundary of a ventral groove on the pygidium (Fig. 6). Only the lateral patches are seen in dorsal view (Fig. 5). The telotroch begins to disappear as the 14th setiger develops.

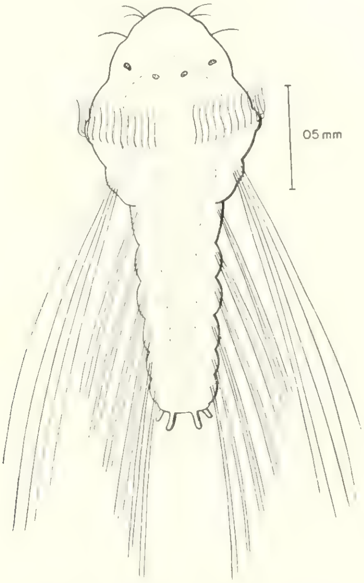

FIGURE 3. Dorsal view of a 7-setiger larva. Telotroch omitted. Figures 5 and 6 are drawn to the same scale.

Prominent gastrotrochs, arranged in four patches across setigers 4 through 9, are present in all planktonic stages (Figs. 4, 6 and 7). Thinner, shorter, inconspicuous cilia may be seen ventrally on segments 1, 2 and 3 of some larvae. Nototrochs are absent in pelagic larvae of this species. Four stiff sensory cilia are present on the prostomium of all pelagic larvae.

Palp buds arise in the 9-setiger stage as small dorsolateral outgrowths anterior to the prototroch (Fig. 5). Although triangular in shape initially, the palps elongate and become highly mobile by the 12-setiger stage.

Branchial *Anlagen* appear in the late 9- or early 10-setiger stage, dorsal to the notosetae on setiger 1. In the 11-setiger stage, branchiae are longer than the palps. However, by the time setiger 13 is added, the palps are approximately twice the length of the branchiae. Branchial ciliation and apical tufts of sensory cilia arise in the 10- to 11-setiger stage.

The collar on setiger 2 becomes apparent on the 11-setiger stage.

Pigmentation of pelagic larvae is restricted to a red-brown line at the base of the prototroch, a similarly colored area on the pygidium, and a pale blue-green region at the base of the swimming setae on setiger 1.

The size and number of anal cirri are variable (Figs. 1 to 7). Although four is the most frequently encountered number, the cirri vary from zero to four. There seems to be no relationship between size or number of anal cirri and the stage of development. Anal cirri begin to disappear during metamorphosis.

III. Metamorphosis and the Length of Larval Life

Metamorphosis is an orderly sequence of events that is completed within a period of 24 hours to more than two weeks, depending upon the stage of larval development and the presence of a suitable substratum. Metamorphosis begins as the larva settles and crawls over and between the particles of sediment. Any remaining swimming setae are lost during the process of the larva's crawling. A few sand grains adhere to the body wall and are carried along with the larva. As other grains are added, a stationary tube is formed within which the larva can move freely. Concomitant with crawling and tube formation is the gradual loss of the prototroch and then the telotroch. As these cilia disappear, the rate of development of branchiae and palps increases. Loss of the telotroch marks the end of the transition from free-swimming larva to benthic form.

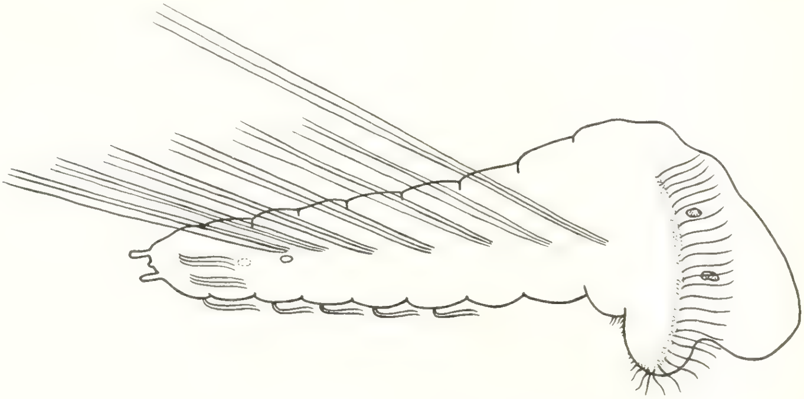

Figure 4. Lateral view of a 7-setiger larva. A crotchet can be seen developing internally in setiger 8. Sensory bristles omitted.

An experiment to test the influence of sediment upon metamorphosis was performed as follows. On August 5, 1963, a female *S. benedicti* was stimulated and 15 larvae were released. These larvae were of 7-setiger size but had not completed the setation and segmentation of the 7-setiger stage. That is, they were at a stage between those shown in Figures 2 and 3. The larvae were divided into four funnels: two with sediment and two without sediment, as controls. On August 8, larvae in the funnels containing sediment had 9 well-developed setigers and had the setae of the 10th partially formed. Only the ventral cilia of the prototroch remained; some of the telotroch cilia had disappeared; and the palps and branchiae were well developed. Metamorphosis was near completion. Larvae in the control funnels were identical in setation to those in the funnels with sediment. However, both prototroch and telotroch were fully formed, and neither palps nor branchiae had begun to develop. By August 12, larvae in the controls had attained the 11-setiger stage and had well-developed palps and branchiae. The controls had developed no farther by August 19 and the experiment was terminated.

The youngest developmental stage that reached metamorphosis successfully was the late trochophore (Fig. 2). In this instance, a brood of 11 late trochophores was released by a stimulated female on August 12, 1963. Larvae from this brood were divided into two groups: one with sediment and one without sediment. Those provided with sediment had reached the 10-setiger stage and had completed metamorphosis by August 19. Those maintained without sediment disintegrated prior to metamorphosis.

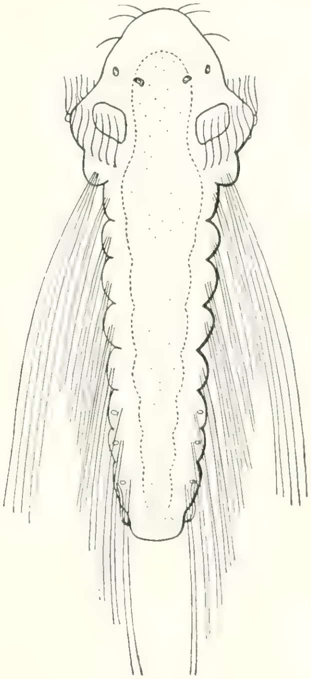

FIGURE 5. Dorsal view of a 9-setiger larva.

Metamorphosis accelerates the development of palps and branchiae. When, for example, 11-setiger stages from the same larval brood are compared, those having metamorphosed exhibit markedly larger palps and branchiae than unmetamorphosed larvae maintained in vessels without sediment.

All larvae, which were released by females in the laboratory and which were provided with sediment soon after liberation, began metamorphosis at the 9-setiger stage and had completed metamorphosis by the 10-setiger stage. Nine- to 13-setiger *S. benedicti* larvae isolated from plankton samples metamorphosed readily

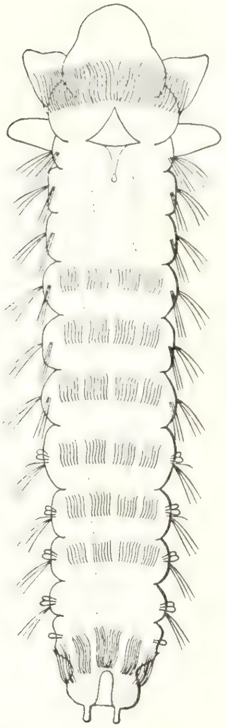

Figure 6. Ventral view of an 11-setiger larva. Sensory bristles and ciliation of palps, branchiae and oral region omitted.

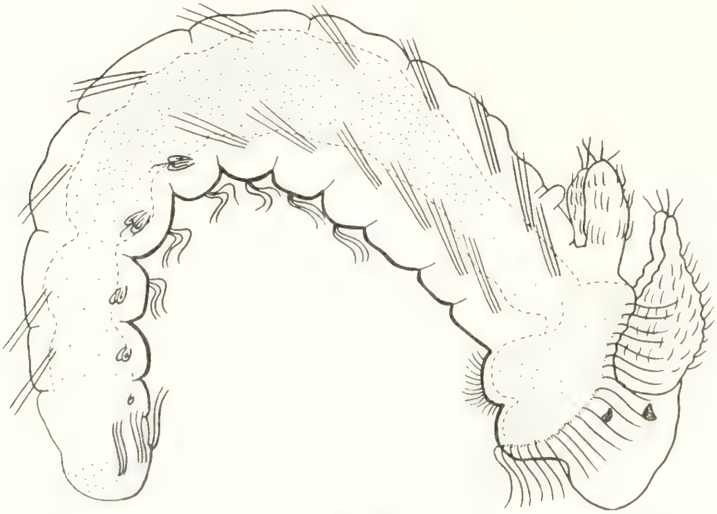

Figure 7. Lateral view of an 11-setiger larva. Sensory bristles omitted.

when they were placed in rearing vessels containing sediment. When similar isolates were maintained without sediment, the larvae would usually reach the 13-setiger stage before disintegrating. Only two specimens metamorphosed successfully in the absence of sediment. In both cases, metamorphosis coincided with the addition of setiger 14.

DISCUSSION

Both larvae at the time of hatching and eggs within females showed the characteristic blue-green coloration reported by Campbell (1957). Shortly after hatching, all coloration is lost except a small area at the bases of the swimming setae on setiger 1.

Several differences exist between the results of the present study and those reported by Campbell (1957). She noted that larvae, when released from the female, had three to four segments; had well developed prototrochs and telotrochs; and had serrated swimming setae, two pairs of red-brown eyes, and four anal cirri. In the present study, however, larvae first attained a length approximating that of a 7-setiger larva before a telotroch was formed or before setation or segmentation took place. It was also found that, unless disturbed, a female would retain larvae until the 9-setiger stage. These observations are supported by the size of *Streblospio* larvae found in the plankton of the Mystic River Estuary. Of the thousands of *Streblospio* larvae observed in plankton samples taken during two summers of study, none were found to have less than 7 setigers. The liberation of 3- and 4-segment stages reported by Campbell (1957) could be explained by rough handling of adults or by differences in reproductive behavior of this species in the two areas.

The spawning season of *Streblospio benedicti* in the Mystic River Estuary is from June to October. During this period the sexes can be distinguished if the females are ovigerous or if the pale, milky posterior region of ripe males is discernible. The sperm is of the aberrant type, a characteristic in keeping with its unusual mode of reproduction (Franzen, 1956).

SUMMARY

1. The larval development of the larviparous spionid polychaete, *Streblospio benedicti*, is described. Specimens were obtained from adults maintained in the laboratory and from plankton isolates. Early and late trochophores, 7-, 9- and 11-setiger stages are illustrated.

2. Undisturbed adult females retain larvae until the 9-setiger stage. If stimulated, however, the female will release younger stages. Under laboratory conditions, late trochophores and all later stages can be reared through metamorphosis. Larvae can metamorphose once they acquire 9 setigers, but they can delay metamorphosis until the 13-setiger stage in the absence of a suitable substratum. Rarely will larvae metamorphose in the absence of sediment. Pelagic life is usually three days or less but can be prolonged for at least two weeks.

3. Seven- to 13-setiger *S. benedicti* larvae occur in the plankton of the Mystic River Estuary from June to October. Nine- and 10-setiger larvae are encountered most frequently.

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THE REACTION OF TWO STARFISHES, PATIRIA MINIATA AND ASTERIAS FORBESI, TO FOREIGN TISSUE IN THE COELOM [1]

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The reactions of invertebrate animals to invasion of the body by foreign materials are not clearly understood, largely because relatively little attention has been focused on this aspect of invertebrate biology. Phagocytosis appears to be the most common defense (Cantacuzène, 1923; Huff, 1940) and has been described by many workers, among them Durham (1888, 1891), Metchnikoff (1891), Kindred (1921), Lison (1930), and Boolootian and Giese (1958). These authors and others note that large objects, such as clumps of carmine which cannot be ingested by individual phagocytes, are generally encapsulated or surrounded by layers of amebocytes. Very little direct work has been done on the specific reactions of invertebrates to large implants, however; the results of Labbe (1929) working on molluscs, Cameron (1932) working on earthworms, Salt (1957) working on insects, and Triplett, Cushing and Durall (1958) working on sipunculid worms, are perhaps typical and suggest that encapsulation may be a general invertebrate reaction toward all foreign objects too large to be phagocytized.

Triplett, Cushing and Durall note that the sipunculid worm, *Dendrostomum zostericolum*, discriminates between pieces of sipunculid and sea anemone tentacles introduced into the coelom, for although both are encapsulated, the sea anemone tissue is killed whereas the sipunculid tentacles are recovered from their capsules alive. The worms are evidently incapable, however, of distinguishing between pieces of sipunculid tentacle of homologous and heterologous origin. The data of Cushing (1957), who made autologous and homologous grafts of eyes to the female portions of the gonads in the scallop, *Aequipecten irradians*, suggest that the hosts are slightly more tolerant of autologous than of homologous grafts, but Cushing points out that his results are preliminary and need extension and confirmation. Anderson (unpublished) performed a brief series of exploratory studies in which he introduced pieces of pyloric caecum from the starfishes, *Pisaster ochraceus* (Order Forcipulata) and *Henricia leviuscula* (Order Spinulosa) into the coelom of another starfish, *Patiria miniata* (Order Spinulosa). He was able to recover the *Pisaster* caecum in apparently healthy condition after ten days in the foreign environment. The *Henricia* caecum, on the other hand, was found to have undergone almost complete disintegration. These results are only preliminary, but they present a contrast to the general pattern of encapsulation described above. They also serve to illustrate the inconsistency of reactions in invertebrate groups, for the *Pisaster* tissue, which was tolerated, is less closely related to the host (in a different order) than the *Henricia* caecum, which was rejected.

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The present study was initiated as a further preliminary exploration of the nature of the reactions of starfishes to foreign tissue in the coelom, in the hope of determining whether the host distinguishes between the tissues of donors of different degrees of relationship, and how the foreign tissues are treated. For many reasons, the asteroids serve as excellent experimental material in transplant studies. The coelom is large and easily reached by knife and hypodermic needle. The coelomic fluid is similar to sea water in composition and tonicity, and transplants may be handled or held in ordinary sea water for considerable periods of time without being damaged. For the same reason, leakage of sea water through the implantation incision in the host body wall does not greatly alter the internal environment of the animal. The animals are generally hardy and regenerate easily. They can withstand the loss of pieces of body wall or of viscera, and they heal experimental incisions well. The pyloric caeca, which are suspended in the coelom by mesenteries, provide excellent tissue for transfer. Caecum tissue is relatively active metabolically and shows a high rate of molecular turnover (Ferguson, 1964a, 1964b) so that its presence has a considerable effect on the composition of the coelomic fluid. It is easily removed from the donor. Above all, it is quite distinctive histologically; in addition to classical accounts, more recent descriptions of normal histology have been provided for *Asterias forbesi* (Anderson, 1953) and *Henricia leviuscula* (Anderson, 1960, 1962). Hence, it is possible to determine by histological study whether a sample seems to be normal and functioning, or to detect changes that may have taken place as a result of sojourn in a host animal.

The general questions toward which this study was directed were the following:

(1) Does a host starfish distinguish between implanted pieces of pyloric caecum from donors of different degrees of relationship?

(2) What is the nature of the animal's reaction to foreign tissue, and by what means (if any) are large pieces removed from the coelom?

(3) What effect, if any, does sojourn in a strange environment have upon tissue of different donors?

The author would like to express her sincere gratitude to Dr. John M. Anderson for his invaluable aid and advice during the course of the study, and for his help in the preparation of the manuscript and the photographs.

MATERIALS AND METHODS

The problem involved introducing pieces of pyloric caecum of one animal (donor) into the coelom of another (host) and determining the fate of such transplants. The host species used were *Patiria miniata,* obtained from the central California coast, and *Asterias forbesi,* obtained from Woods Hole, Massachusetts. Fairly small specimens, ranging from one to three inches in diameter, were used because they required relatively little aquarium space and their body walls were thin and easy to cut. Donors included members of the host species and of two others, *Asterias vulgaris* and *Henricia sanguinolenta*, both obtained from Woods Hole. The animals were kept in running sea water at the Marine Biological Laboratory at Woods Hole, where most of the experiments on *Asterias* were done, and in tanks of circulating refrigerated sea water at Cornell University, where the balance of the experiments on *Asterias* and all those on *Patiria* were performed.

Before being subjected to any experimental treatment, both hosts and donors were first relaxed in a solution of 7.5–8% $MgCl_2$ in tap water. Pyloric caecum was then removed from the donors and, in order to distinguish it from the caecum of the host, was stained for at least two minutes in 0.01% methylene blue in sea water, to which had been added 0.03% egg albumin to reduce the toxicity of the dye (Chalkley and Park, 1947). The fact that homologous tissues stained in this way were not rejected by the host indicates that this treatment does not significantly alter the chemistry of the transplants. Implanted tissues retained the blue color for more than six weeks and thus were easy to identify in gross dissection; the stain was completely removed, however, by subsequent histological procedures. The staining step was omitted when *Henricia* was the donor, for the caecum of this species is brilliant red-orange in color and is easy to identify without any special treatment.

The tissue was trimmed and injected by means of a large-bore hypodermic needle (#16), which was inserted into the ray opposite the host ray. In this way, the body wall of the host ray was left intact and the tissue was not likely to slip out immediately upon implantation. The cardiac stomach, and perhaps the pyloric as well, suffered some trauma when the needle passed through it, but its many folds and great elasticity served to occlude any openings through which the tissue might escape. With the needle in position, the trimmed donor caecum was drawn into the syringe with a small amount of sea water, the syringe was connected to the positioned needle and the tissue expelled into the coelom of the host. Care was taken not to introduce air bubbles into the coelom during this process. The advantage in first positioning the needle and then attaching the syringe lay primarily in the fact that there was less chance of macerating the tissue if it had to pass through the needle only once.

Two rays of an individual were generally used as host rays, and each was considered a separate experimental case. When an autologous transplant was being made, one of the rays opposite that intended to be the host ray was chosen as the donor ray, and the tissue was removed from it through a slit made in the aboral wall. The needle could later be inserted into the same incision. In some of the smaller specimens of *Asterias*, where it was difficult to slit the body wall accurately because of the small area of the aboral surface of the rays, the tip of the donor ray was cut off and the caecum removed through the open end. Thereafter, the procedure for the autologous transplants was identical to that for the others.

The hosts were examined daily for at least a week after implantation. Presence of edema, autotomy of host rays, and such signs of morbidity as shedding of patches of body wall or development of excessive flaccidity were noted. In addition, the dermal branchiae and oral and anal areas were checked for indications of the elimination of implanted tissue. At the end of a week (or longer in some cases), the hosts were dissected after relaxation in $MgCl_2$, and the disposition of the transplants noted. If the tissue was recovered, it was fixed in Bouin's solution for at least 24 hours (48, if it included calcareous elements), and after washing in 80% alcohol, was dehydrated, cleared, and embedded in paraffin. Sections were cut at 10 μ and stained with Mallory's phosphotungstic acid hematoxylin, which differentiates muscle, connective tissue, cell membranes, and secretion granules, and were examined for evidence of growth, necrosis, and other changes. Control pieces of caecum that had been passed through the needle without being implanted were similarly fixed, sectioned, and stained, in order to estimate the damage that might be caused to the tissue by the injection procedure.

RESULTS

a. Implantation experiments

When the animals were examined and dissected, it was found that the disposition of the donor tissue tended to fall into one of four categories, scored as follows:

(a) "Leaking"—The transplant caecum began to pile up in the dermal branchiae of the hosts, usually appearing here within one to three days after implantation. The distal halves of the branchiae, which had become swollen with donor tissue, often rounded up and pinched off from the bases, in effect undergoing autotomy. In other cases, the branchiae ruptured without autotomizing, so that the tissue simply passed out through the distal openings. In a few instances, where several adjacent branchiae had ruptured simultaneously, actual rifts in the body wall were formed, through which masses of implanted tissue could be seen emerging. In any case, when the hosts were dissected at the end of the week, the donor tissue was recovered, if at all, as a few small pieces projecting into the coelom from the bases of the branchiae.

(b) "Not recovered"—In many cases, the transplant was nowhere to be seen when the host was dissected. Parts of host caecum or body wall were stained with methylene blue, however, indicating that the donor tissue had been successfully injected into the coelom and had remained there long enough to impart some of its blue coloring to the surrounding host tissue. *Asterias* transplants tended to transfer more of the stain than *Patiria* transplants. It seems reasonable to assume that the tissue had in fact been eliminated, but by some route other than through the dermal branchiae.

(c) "Recovered"—The donor tissue was found apparently intact, either floating free in the coelom, or attached to the host body wall or pyloric caeca.

(d) "Lost"—When it was impossible to trace the disposition of the tissue, *i.e.*, when there was no recovery or evidence of leakage, and no staining of any host tissues could be noted, it was assumed that the tissue had not been successfully introduced into the coelom, and the data for that case were discarded. Into this category were also placed animals that had become moribund or had autotomized the host ray(s).

The first series of experiments involved transplanting pieces of homologous or heterologous pyloric caecum to determine the reaction of the hosts to transplants in general, and to see whether there were differences in the treatment of homologous and heterologous tissues. Table I presents a synopsis of the results of this series.

The figures for donor *Asterias* include both *A. forbesi* and *A. vulgaris*, between which no distinction was apparently made by either *Asterias* or *Patiria* hosts. These data indicate that both host species tend to tolerate their own tissue and to eliminate that of the other species. In order to confirm these observations, several double implantations were made, with each host receiving one homologous and one heterologous piece of caecum. Table II summarizes the results of these experiments. In the case of the single piece of *Henricia* caecum which was not recovered, the initial injection was probably unsuccessful.

TABLE I

Results of experiments demonstrating host reaction to single implants of pyloric caecum

Host	Donor	Leaking	Recovered	Not recovered	Total
Patiria	*Patiria*	3	29	0	32
	Asterias	0	1	13	14
	Henricia	9	1 (in frags.)	0	10
Asterias	*Asterias*	0	11	0	11
	Henricia	7	0	0	7

The results of both sets of experiments are mutually consistent and present evidence of what seems to be definite discrimination between homologous and heterologous transplants. These figures also demonstrate that the same animal can retain one transplant while eliminating another.

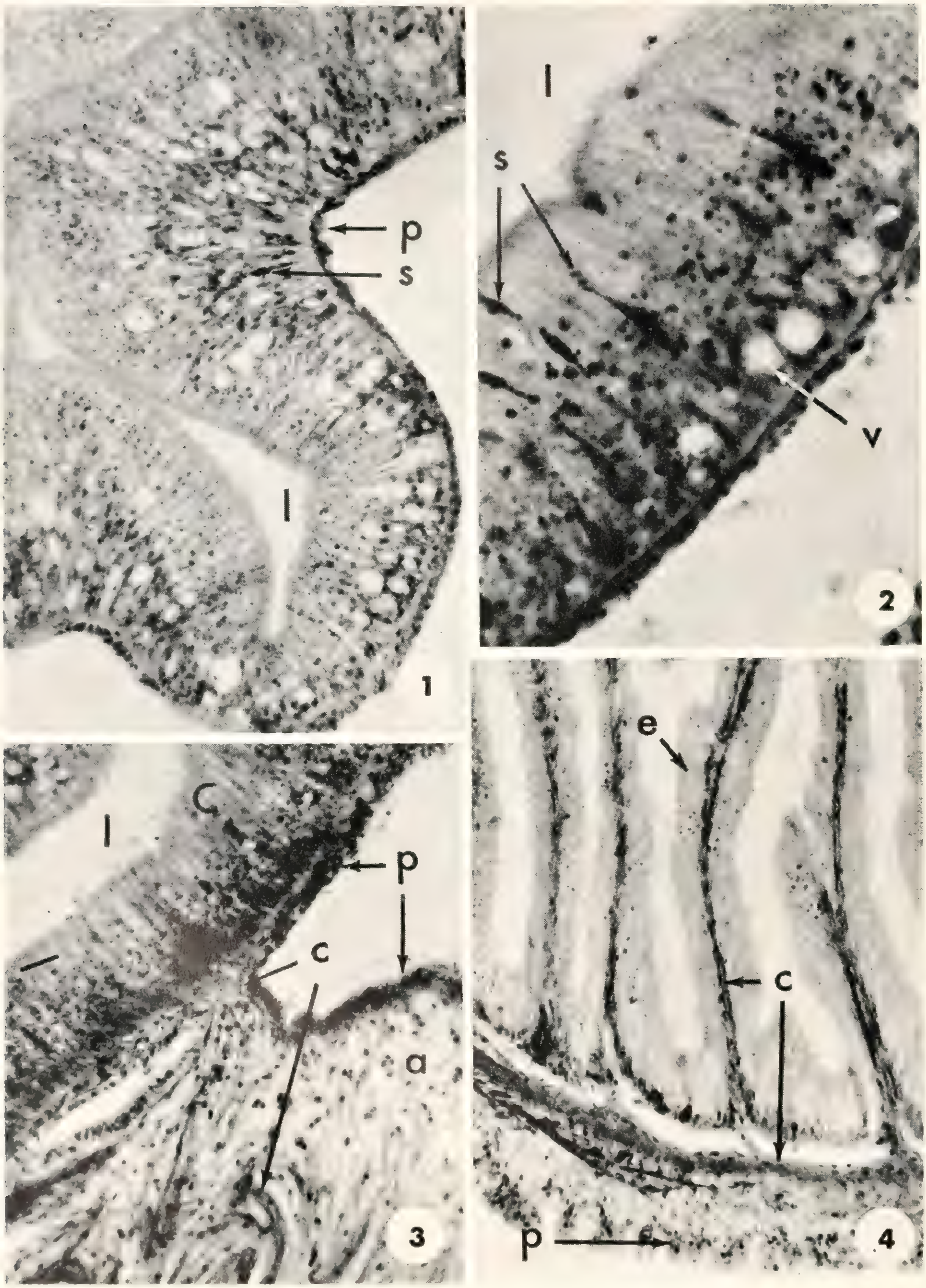
There are evident contrasts between the modes of elimination of *Asterias* and *Henricia* caecum by both *Patiria* and *Asterias*. *Patiria*, though it eliminates *Henricia* caecum through the branchiae, may dispose of *Asterias* tissue by pushing it back into the cardiac stomach, for on several occasions, transplanted tissue was recovered projecting from the mouth. *Asterias* also eliminates *Henricia* caecum through the branchiae. Unfortunately, transportation difficulties made it impossible to obtain an adequate supply of *Asterias* in Ithaca, and I was unable to determine how this species reacts to *Patiria* implants.

TABLE II

Results of experiments determining whether a single host discriminates between simultaneously implanted homologous and heterologous caecum

Host	Donor	Leaking	Recovered	Not recovered	Total
Patiria	*Patiria*	0	4	0	4
	Asterias	0	0	4	4
Asterias	*Asterias*	0	11	0	11
	Henricia	9	1	1	11

To determine whether tolerance of homologous material in the coelom extended to tissue taken from other parts of the body, observations were made on homologous transplants of tissue other than pyloric caecum. Eight *Patiria* host rays were im-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FIGURES 1-4.

planted with homologous rectal caecum, an organ normally found in the aboral region of the central disc. All eight transplants were recovered in apparently healthy condition, and in one case, the implanted rectal caecum was joined to an adjacent pyloric caecum of the host.

On other occasions, recovered pyloric caecum transplants, especially older ones which had spent three or more weeks in the host coelom, had fragments of ossicles embedded within them. The sources of these fragments are uncertain; perhaps they had been picked up and carried in by the tip of the needle during the implantation procedure. This retention of both rectal caecum and ossicle fragments, despite their considerable physical differences from pyloric caecum, supports the view that elimination cannot be based on physical differences alone. It also suggests that the animal is not sensitive to the presence of tissue elements in the wrong places in the body.

b. Histological observations

Normal pyloric caecum is composed of an outer peritoneum of cuboidal cells, layers of muscular, connective and nervous tissue, and an inner epithelium of tall flagellated columnar cells, this layer containing mucous gland cells and secretory cells producing vesicles and granules (Anderson, 1953; see also Fig. 1). Examination of pieces of control caecum which had been passed through the needle before fixation revealed areas of tissue normal in every respect, interspersed with places where the tissue had been broken and abraded by the injection process.

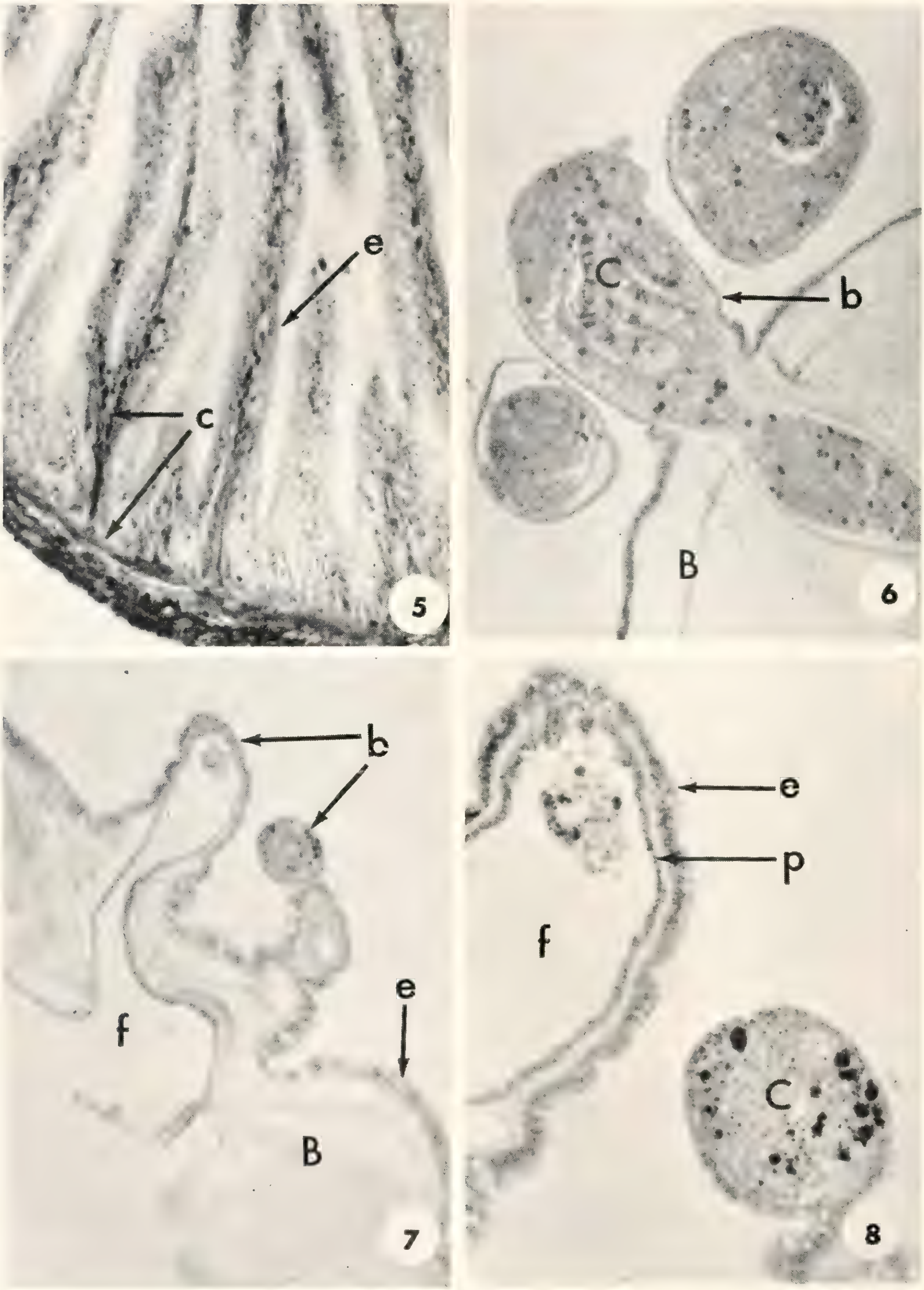
Both *Patiria* and *Asterias* pyloric caecum transplants recovered after one to five weeks in homologous hosts generally showed the same mixed appearance as the injected and fixed controls, healthy areas alternating with damaged ones. The sound areas (Fig. 2) were generally indistinguishable from normal tissue. The presence of prominent secretion granules in the recovered caecum (Fig. 2) indicates that metabolic activities were continuing at the time of fixation. There were few amebocytes associated with the intact areas. Damaged areas, on the other hand, showed changes, particularly evidence of necrosis and disorganization associated with amebocytes. The amebocytes were often rounded and filled with what appeared to be cellular debris, including recognizable secretion granules. Whether these phagocytes came from the donor, the host, or both could not be determined.

FIGURE 1. *Patiria,* normal pyloric caecum. Many of the tall columnar epithelial cells contain secretion granules and vesicles. × 260. l, lumen of caecum; p, peritoneum; s, secretory cell with granules.

FIGURE 2. *Patiria,* pyloric caecum recovered after four weeks in a homologous host. There is no evidence of damage or necrosis; the secretory cells show evidence of continuing normal metabolic activity. × 520. l, lumen of caecum; s, secretory cell with granules; v, vesicle.

FIGURE 3. *Patiria,* pyloric caecum with attached mass of amebocytes and connective tissue recovered from a homologous host after four weeks. The mass of new cells is associated with damaged areas of caecum (not shown) but has extended to join the healthy caecum at left. The connective tissue fibers in the new tissue are continuous with those in the donor caecum. Peritoneum, also continuous with that of the donor caecum, covers the amebocyte mass. × 260. a, amebocyte mass; C, donor caecum; c, connective tissue fibers; l, lumen of caecum; p, peritoneum.

FIGURE 4. *Patiria,* normal rectal caecum. The inner epithelium is thrown into folds supported by connective tissue partitions. × 260. c, connective tissue fibers; e, epithelium; p, peritoneum.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Figures 5–8.

Recovered transplants were found joined to the host caecum or body wall on several occasions. In some specimens, connections were made at several points. The more tenuous of these links appeared to be established by amebocytes alone, while more substantial bridges showed connective tissue fibers in addition. It seems probable that the amebocytes established the first bridges, with connective tissue fibers invading later.

Some of the older transplants (four or five weeks) had masses of amebocytes and connective tissue filling in the gaps or folds in the implant (Fig. 3). This growth was often quite extensive and gave the impression of rounding out the contours of the transplant. In some cases, peritoneal epithelium could be seen around the mass of new cells (Fig. 3). The development of the amebocyte masses usually began on the borders of the damaged sections, occasionally extending to the healthy areas as well. Where ossicles were embedded in the transplant, they were also associated with amebocytes and connective tissue, and appeared to be firmly joined to the surrounding caecum. In no case, however, did the recovered homologous tissue give the impression of undergoing encapsulation, for areas not obviously damaged were often free of amebocyte activity.

Normal rectal caecum shows the same layers as normal pyloric caecum, but the inner epithelium is greatly folded, each fold being supported by inward extensions of the connective tissue layer (Fig. 4). Rectal caecum recovered after a week (Fig. 5) was for the most part indistinguishable from the normal caecum, although it did show some local damage associated with amebocyte activity and connective tissue formation similar to that described in the recovered pyloric caecum.

One case of encapsulation was observed, involving a small fragment of *Asterias* transplant recovered from a host *Patiria*. Sections showed a small core of extremely disorganized donor tissue with a few scattered secretion granules remaining, surrounded by amebocytes. There was no evidence of connective tissue formation.

Examination of sections of body wall in which the dermal branchiae were eliminating *Henricia* caecum showed interesting details. The branchiae were rounded and swollen at their tips, which either ruptured (Fig. 6), or pinched off (Figs. 7, 8). The walls of the swollen parts were stretched quite thin compared with the more typical branchiae (Fig. 8); the columnar epidermis appeared almost cuboidal, the connective tissue layer was greatly attenuated, and the peritoneum was vague and indistinct. It was impossible to distinguish the muscle layers at all.

Figure 5. *Patiria*, rectal caecum recovered from homologous host after one week (slightly oblique section). This tissue is virtually indistinguishable from the normal rectal caecum (Fig. 4). × 260. c, connective tissue fibers; e, epithelium.

Figure 6. *Henricia* caecum being eliminated through ruptured *Asterias* branchia. (The section is not quite parallel to the long axis of the branchia.) This branchia and the ones on either side are packed with *Henricia* caecum. Note the expansion of the tissue already out of the branchia. × 90. B, body wall; b, branchial wall; C, *Henricia* caecum.

Figure 7. *Asterias* branchia eliminating *Henricia* caecum. (Section is slightly tangential to the long axis of the branchia.) The tip of the branchia containing the *Henricia* caecum is separated from the rest by a prominent constriction. Note the wrinkling of the base of the branchia. × 90. B, body wall; b, branchiae; e, epidermis; f, coelom.

Figure 8. Detail of Figure 7. Note how the wall of the autotomizing portion of the branchia has been stretched. (Compare with the more typical branchia at left.) × 260. C, *Henricia* caecum; e, epidermis of branchia; f, coelom (cavity of branchia); p, peritoneum.

In contrast, the bases of the branchiae, below the point of constriction, appeared quite normal histologically, except that they were slightly wrinkled transversely (Fig. 7) as if either circular or longitudinal muscles or both were somewhat contracted. Where the branchiae had ruptured, *Henricia* caecum could be seen squeezing through the channel provided by the branchial walls and escaping to the outside (Fig. 6). The caecum in the branchiae was not in fragments, but rather gave the impression of being folded and packed into the lumen of the organ (Fig. 6). None of the *Henricia* caecum appeared to have unusual numbers of amebocytes associated with it, although it was clearly being actively eliminated.

Discussion

The results of these experiments suggest that there are at least three different means of elimination of foreign materials from the asteroid coelom. The first, amebocytic attack, was expected from accounts in the literature. The second, elimination of tissue through rupturing or autotomizing branchiae, was somewhat of a surprise, for although Lison (1930) reported branchial autotomy in response to the presence of large clumps of amebocytes in the branchiae, I am aware of no other report of branchial elimination of pieces of tissue. The third method, that still undetermined means by which *Patiria* eliminated *Asterias* pyloric caecum (possibly through the cardiac stomach, although I have no histological evidence that this was the case), was also not predictable on the basis of previous reports of elimination of material.

Phagocytosis of particulate matter may be demonstrated easily in both *Patiria* and *Asterias* by injection of a carmine suspension into the coelom; the branchiae shortly become filled with amebocytes bearing ingested carmine particles. However, amebocytic attack and invasion cannot be responsible for the branchial elimination of *Henricia* caecum, for the tissue in the branchiae remains in a relatively intact state and has few amebocytes associated with it. Nor does encapsulation by amebocytes seem to be a common response to the presence of large foreign bodies in the coelom, for it was observed only once during the whole study.

It was not possible to determine at this time just why *Henricia* caecum was selectively eliminated through the branchiae by both hosts. The mechanisms involved might depend upon physical or chemical differences, or both, between this caecum and those of *Asterias* and *Patiria*. In any case, this means of elimination seems to be fairly important to both hosts; at least it was consistently employed.

The filling and rupturing of the branchiae must require a considerable amount of force, perhaps more than can be accounted for on the basis of ciliary currents alone. The musculature of the branchiae, consisting of both longitudinal and circular fibers, might be aiding the movement of the tissue to the tip, perhaps by peristaltic action. The longitudinal muscles normally retract the branchiae in response to external disturbances, and the circular muscles are antagonistic to the longitudinal muscles. However, the fact that an autotomizing branchia may be pinching in strongly at one level alone and nowhere else along its length implies rather specific control over local contraction, which in turn suggests that peristaltic movement is at least theoretically possible. The wrinkled appearance of the autotomizing branchiae below the point of constriction, as seen in Figure 7, might indicate that such contraction was going on at the time the tissue was fixed.

In addition to eliminating material through the branchiae, *Asterias* can quickly and easily autotomize whole rays. *Patiria* cannot autotomize easily, but the efficiency with which it evidently eliminated the *Asterias* transplants suggests that it is well protected despite its inability to shed rays. To determine whether *Asterias* can also eliminate material through the cardiac stomach (if this is in fact how *Patiria* eliminates the *Asterias* tissue) or whether this species relies entirely on use of the branchiae and on autotomy requires further experimentation.

The question of the level (*i.e.,* tissue, organ, or higher) at which these elimination mechanisms are operating is experimentally beyond the scope of the present study, but it presents possibilities for future investigations. Elimination through the branchiae, for example, may be a complex process; it remains to be determined whether it is an automatic local reaction to the presence of anything in the branchiae, or whether the process is selective and under the regulation of the animal as a whole. If the latter is the case, one might well consider it an integrated response. It is also important to remember that local and general effects may be occurring simultaneously and that demonstration of the one does not preclude the existence of the other.

The role of the amebocytes associated with the damaged areas of the recovered caecum may be a multiple one. It is unlikely that they are encapsulating the implant, walling it off from the coelom by forming a shell around it, as this process was found only once in this series of experiments. They are almost certainly engaged in cleaning up cellular debris in these damaged areas. They might also be participating in regeneration of fragmented caecum. Anderson (1962), studying the regeneration of pyloric caeca in *Henricia leviuscula,* discusses the possibility that the amebocytes might actually be differentiating and supplying cells for the new caecum. Here, also, more work must be done to understand what is taking place.

The results of the present preliminary study may or may not indicate that responses of an immunological nature are evoked by tissue implants. The response of the host does seem to vary in relation to the source of the implanted tissue. No distinction was made (within the framework of present experiments) between autologous and homologous tissue, or between tissue of two closely related species, *Asterias forbesi* and *Asterias vulgaris.* On the other hand, there was discrimination between members of two different families in the same order (*Henricia* and *Patiria*), and between members of two different orders (*Asterias* and the other two). These latter results are in agreement with those of Anderson (unpublished) on the rejection of *Henricia* caecum by *Patiria.* They are contrary, however, to his findings on the *Pisaster* implants, which were evidently tolerated by *Patiria.* This discrepancy is unexplained and might conceivably be another example of the "disconcerting inconsistency" mentioned by Cantacuzène (1923) as characteristic of invertebrate reactions.

The mechanism of distinction is obscure, but as metabolizing caecum is undoubtedly exchanging molecules with the coelomic fluid (Ferguson, 1964a, 1964b), the host may be reacting to the presence of atypical molecules in the coelomic fluid. On this basis, also, the distinction between "tissue" and "general" levels of reaction becomes a pertinent question.

Much more work remains to confirm and extend these results. No attempt was made to follow the progress of any of the transplants for longer than five weeks. It may be that all foreign tissue is eventually rejected or perhaps encapsulated if enough time is provided, although the experimental data suggest that this is probably not the case. Other donor-host combinations should be tried to determine how specific and how consistent these reactions actually are and where the lines are drawn between tolerance and rejection. The role of the amebocytes needs clarification with respect to such questions as whether they are only removing debris, or are also taking part in regeneration of tissue in the damaged areas of caecum. Until these questions are answered, the precise bases of these reactions toward foreign implants will remain unknown.

SUMMARY

1. Two starfishes, *Patiria miniata* and *Asterias forbesi,* were found to discriminate between coelomic implants of homologous and heterologous pyloric caecum obtained from donors of these species and of two others, *Asterias vulgaris* and *Henricia sanguinolenta.*

2. Homologous transplants were recovered from the hosts one to five weeks after implantation and were found to be generally normal in histological appearance, although there was some growth of connective tissue and amebocyte masses in areas which had probably been damaged during the implantation procedure. Heterologous transplants were eliminated by the hosts within a week of implantation.

3. *Henricia* caecum was eliminated through the dermal branchiae by both hosts. *Patiria,* in contrast, eliminated *Asterias* caecum in a still undetermined way; the tissue may have been transferred from the host ray to the cardiac stomach, and either digested there or passed out through the mouth.

4. Contrary to expectation, amebocytic attack, phagocytosis, and encapsulation were not seen to play an important role in elimination of heterologous transplants, although amebocytes were associated with damaged areas in the homologous transplants. Such amebocytic activity is probably concerned with removal of cellular debris.

5. The results of these experiments may express a general tendency of starfishes to distinguish between implants of donors of different degrees of relationship, or they may represent special cases of discrimination. More work remains to determine how specific these tolerance-rejection thresholds are and how consistently they vary with different host-donor combinations.

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THE EFFECTS OF TEMPERATURE AND SALINITY CHANGE ON SWIMMING RATE IN THE DINOFLAGELLATES, GONYAULAX AND GYRODINIUM[1]

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Because of technical difficulties there have been few definitive studies in which swimming rate of the individual motile microorganism has been measured with accuracy. Wingo and Browning (1951) determined the velocity of *Tetrahymena.* Dryl (1961) has studied the effects of pH change on linear velocity in *Paramecium.* These studies were conducted using conventional photomicroscope techniques. A more sophisticated approach to the investigation of swimming behavior has been taken by Rothschild (1953) and van Duijn and Rikmenspoel (1960) in their studies on sperm; here photoelectronic methods yielded accurate data on velocity, etc.

Davenport, Wright and Causley (1962) have described an electronic tracking technique with which it is possible, with great accuracy and rapidity, to obtain data on linear velocity and rate of change of direction in the individual microorganism under a variety of conditions. The studies to be described were initiated because of the obvious importance of understanding the effects of changes in environmental parameters on the activity of motile phytoplankters. To this end we chose as experimental organisms two dinoflagellates, the littoral *Gonyaulax polyedra* Stein and a to-date unidentified *Gyrodinium.* The former is the well-known causative agent of "red-tides" (Bonnot and Phillips, 1938; Allen, 1946; Connell and Cross, 1950; etc.). Its vertical migration has been studied by Hasle (1950, 1954) and the cyclic nature of its luminescent activity by Haxo and Sweeney (1955, etc.). Our *Gyrodinium* sp. was apparently "selected for" by the particular environmental conditions of a shallow salt lagoon on the University campus which receives the outflow of the Marine Laboratory; it "bloomed" so richly as to contribute to the death of numbers of *Gillichthys* and *Fundulus.* Since certain species of *Gyrodinium* are known to be characteristically denizens of lagoons and salt marshes, we hoped that our form would show different tolerances than the littoral *Gonyaulax.* As shall be seen, this proved to be true.

It was the purpose of these initial studies to establish the effects of temperature and salinity change on the behavior of the two organisms.

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[1] These investigations were conducted under Contract NONR-4222-03 with the Office of Naval Research.

MATERIAL, METHODS

The apparatus [2]

A gated square sweep (scan) of uniform low intensity is generated on a Dumont Type 304H oscilloscope mounted vertically (Fig. 1). The gated sweep is accomplished by a generating circuit which is capable of producing several sweep frequencies. The scan frequency is further controllable by emphasizing a certain section of the generated sweep with accurate regularity. This is accomplished by the incorporation of a blanking circuit which can also be adjusted. By adjusting the interplay between these two controls, as well as the oscilloscope intensity, it is possible to obtain gated scans with intervals of one scan per ten minutes to fifty scans per second.

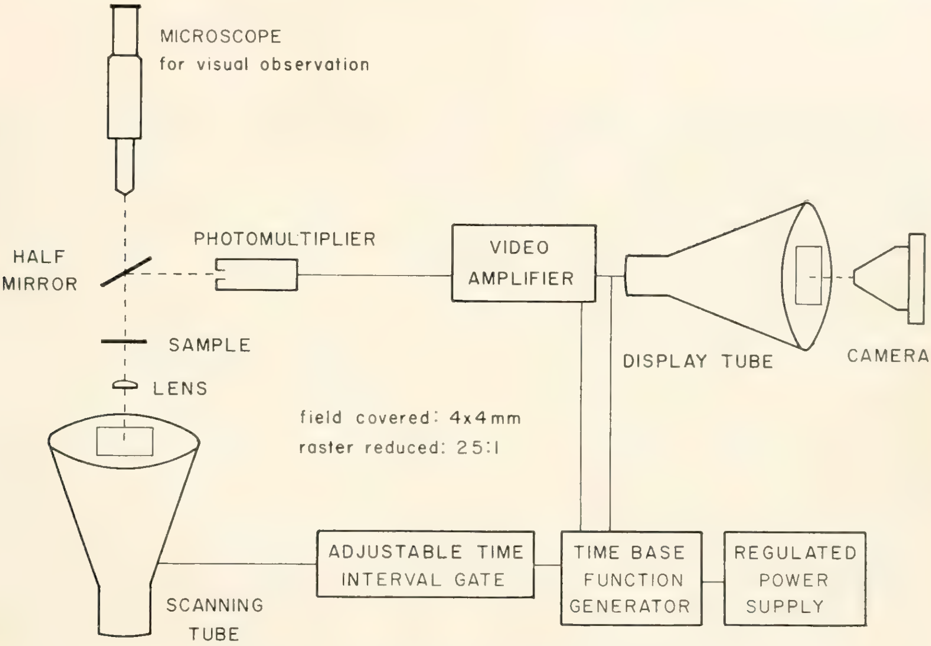
MICROSCOPE
for visual observation
PHOTOMULTIPLIER
HALF MIRROR
VIDEO AMPLIFIER
SAMPLE
LENS
DISPLAY TUBE
CAMERA
field covered: 4x4mm
raster reduced: 25:1
ADJUSTABLE TIME INTERVAL GATE
TIME BASE FUNCTION GENERATOR
REGULATED POWER SUPPLY
SCANNING TUBE

FIGURE 1. Diagram of the scanning apparatus.

The gated scan is transmitted optically to a microscope stage above the oscilloscope *via* a 1.0-mm. camera lens mounted below the stage. The position of this lens can be adjusted by a fine-adjustment so that the scan may be focussed on a prepared slide of the organism to be observed. The area of the test preparation scanned is a 4-mm. square, while the depth of field is less than 0.5 mm. The magnification of the subject may be changed by manipulating the horizontal and vertical sweep controls of the vertically mounted oscilloscope. The field scanned remains the same.

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[2] The authors wish to express their appreciation to Dr. James Moyer and Messrs. Robert King and Eugene Evinger, who cooperated in the construction of the apparatus by Servomechanisms Inc. of Santa Barbara.

The scan is transferred by a mirror to a type 1P21 photomultiplier tube and associated preamplifier unit. Changes in light intensity within the scan, caused by objects (such as moving organisms) between the scan and the photomultiplier tube, are registered by that tube as changes in electrical potential. The potential changes are amplified and transmitted to a second Dumont 304H oscilloscope mounted horizontally. This scope in turn displays a "negative" image of the intensity differences in the generated scan, *i.e.,* the discrete areas of reduced illumination in the original scan are finally displayed as light "blips" on a dark field, each blip recording the organism's position at the moment it is scanned. Resolution of the system is fine enough for it to perceive relatively opaque organisms larger than 15–20 μ, and to distinguish the external morphological features of many forms in the 50–300 μ range. By coupling a Dumont type 450A oscilloscope camera fitted with a Robot "Recorder 24" 35-mm. camera-back to the display scope, track images of moving organisms may be photographically recorded as they occur; a time exposure on Kodak Tri X Pan film records the positional images or blips for a chosen number of scans (Fig. 2). With scan rate as well as power of magnification known, information necessary for linear velocity determination is available. We determine field magnification by photographing, at the beginning of each test series, a calibrated guide of known dimension; for this purpose a lucite slide inscribed with parallel lines 1 mm. apart was used.

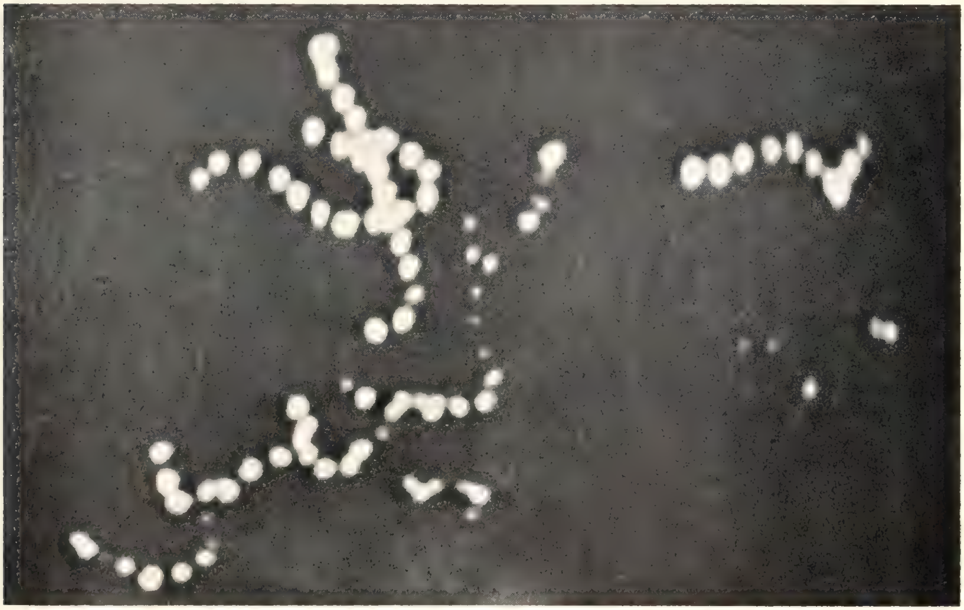

FIGURE 2. Pathways of *Gonyaulax polyedra.*

Data analysis

Films of pathways were projected onto a 1 × 1.5 M. rectangular wall-screen divided into nine equal sections, each of which was numbered. A table of random numbers was used to select the section in which data were collected. All discrete (clearly measurable) pathways falling within or bordering on a chosen section were measured with the aid of a standard planimeter calibrated in centimeters. In practice, a convenient length was selected for measurement, depending upon the velocity of the organism and the consequent scanning rate; as an example, when a scan rate of 5/sec. was used, the distance between the first and last of a series of six blips was measured center-to-center for all chosen pathways, to give distance travelled in a single second. In certain experiments a shorter (or longer) pathway was so measured; when this was done a correction factor was used to standardize distance travelled in one second. Standard procedure was to compute mean distance travelled in one second for a sample of fifty organisms.

The conversion of data obtained to true linear velocity values was accomplished by comparing the dimension of the projected scan of the 1-mm. calibration on the above-mentioned lucite guide with the actual dimension, which gave the power of magnification. In our studies the power of magnification ranged from 360 to 380 ×. This magnification gave accurate readings to the nearest 10 μ, which was sufficiently accurate to allow analysis of statistical significance.

Culturing and scanning techniques

Unialgal cultures of *Gonyaulax* were maintained in the manner described by Haxo and Sweeney (1955) with some modification (Bennett—personal communication).[3] The stock solution consists of 750 cc. filtered sea water, 216 cc. twice-distilled water, 2 cc. 1 *M* KNO_3, 2 cc. 1 *M* K_2HPO_4, 0.1 cc. 0.1 *M* $FeCl_3$, 10 cc. of 1 gm./liter EDTA and 20 cc. of 1 gm./cc. soil extract (autoclaved 15 minutes and filtered). The medium is then autoclaved 15 minutes at 15 lbs. pressure. It is allowed to stand for 24 hours to regain CO_2 balance. It may then be distributed into 125-ml. Erlenmeyer flasks, adding 40 ml. to each flask. The flasks are then inoculated with cells.

Inoculated flasks were placed under continuous illumination furnished by cold white fluorescent tubes with an intensity of 700 foot-candles. The cultures were maintained at 20° C. They reached maximum growth in about three weeks.

The experiments of Miss Bennett enabled us to maintain *Gyrodinium* cultures very successfully in the above *Gonyaulax* medium with the following additions:

To one liter of *Gonyaulax* medium add 10 cc. of the following stock solution:

Thiamine HCL	20.0 mg.
Biotin	0.05
Folic acid	0.2
Inositol	100.0
Ca pantothenate	10.0
Nicotinic acid	0.01
P-aminobenzoic acid	0.1
Twice-distilled water	100.0 cc.

pH to 7.8–8.2 with 0.1 N NaOH and add
Vitamin B_{12}—1 gamma per liter medium.

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[3] The authors wish to express their appreciation to Miss Carrie Bennett of the Dept. of Marine Botany, Scripps Institution of Oceanography, for her help and advice on culture methods.

All experiments were conducted using cultures 14 days old. These were maintained under culture conditions (constant illumination, at 20° C.) during experiments. Drop samples were removed from them, placed on standard micro slides which had been serially rinsed in twice-distilled water, placed in position, scanned, photographed and discarded within a period of ten seconds. Scanning and photographing were done in darkness, the organisms for this brief period being subject only to the light of the scanner. Ambient temperature was 20° C. With one exception to be mentioned, all preparations were of the open-drop type. The rate of evaporation occurring in an open-drop preparation during the testing period was estimated with the aid of a conductivity bridge. The conductivity was determined using a cell which would give a measurement of conductivity with a 0.1-cc. sample of medium (the average size of the exposed drop). Exposed drop samples were made and allowed to stand for times ranging from one second to 15 seconds. With the conductivity measurements obtained from these samples, the amount of evaporation occurring per unit time was determined. The results obtained indicated that no measurable amount of evaporation could be observed for the first ten seconds after the preparation of the slide.

Experiments

Determination of linear velocity

Gonyaulax

Approximately 0.4 cc. of *Gonyaulax* culture was pipetted into a lucite well-slide. The well was circular, with a flat bottom and vertical sides, its dimension being 8.0 mm. in diameter by 1.5 mm. deep. A coverslip was placed over the well. It was then scanned, photographed and discarded. Fifty individual pathways were recorded from such preparations and measured as described above.

From these preparations a mean linear velocity of 250 μ/sec. (with a range from 175 μ/sec. to 325 μ/sec.) was obtained for the *Gonyaulax* cell at 20° C.

Since in the above experiment it was possible to measure linear velocity in the horizontal plane only, a series was conducted to determine velocity in the vertical plane. This was accomplished simply by rotating the entire scanning elements of the apparatus, including the preparation under observation, through 90°. Velocities for the two planes were compared statistically, using a one-way analysis of variance, and found not to be significantly different ($F = 0.96$ n.s.).

Gyrodinium

Mean linear velocity for this form was found to be 319 μ/sec. at 20° C. Here again plane of movement was not a significant factor. The range was similar, being between 220 μ/sec. and 360 μ/sec.

The relation of linear velocity to salinity

Gonyaulax

In order to determine the gross reactions of these organisms to variations in salinity, a series of subjective preliminary observations was conducted.

A series of culture samples was prepared, ranging at 10% intervals from 100% culture medium (28 ‰) to 40% (10 ‰). These samples were prepared from a single stock culture by diluting the appropriate amount of culture medium with twice-distilled water to make a 10-ml. sample of the desired salinity. The desired amount of distilled water was slowly added to the cell-containing medium and stirred gently to insure proper mixing. The beakers containing the series of dilutions were covered with Parafilm, placed under culture conditions and left undisturbed for a period of not less than four hours. In the examination of activity under hypersaline conditions, similar procedure was employed with the substitution of 200% sea water for twice-distilled water. Observations were then conducted on each series over a period of twelve hours.

Immediately following sample preparation, the majority of the cells were observed to cease normal activity and settle to the bottom of the container. Controls differed from experimental samples in that the time necessary for recovery when one transferred cells from a normal culture into "cell-free" culture medium was a matter of a few minutes only. In such controls the proportion of cells which settled out, even for a brief period, was less than when salinity differed from the medium.

At the end of the four-hour "acclimation period" motility was observed in the samples from 40% to 150% medium. In the 150% samples there were more motile cells observed at the end of 12 hours than after four hours. In all other samples, the total number of motile cells did not increase after the four-hour period.

With the information at hand from the above subjective observations, a test series was prepared in a similar manner and scanned after the four-hour acclimation period. The results of these experiments may be seen in Figure 3. The plot in the figure represents the best fitting ($F = 1201.73$) mathematical relationship between linear velocity and salinity for these data.

To compare the behavior of the *Gonyaulax* cell in varying concentrations of culture medium with its behavior in varying concentrations of sea water, the following test was conducted. One hundred ml. sea water were added to 25 ml. culture in a filter funnel lined with Whatman #5 filter paper. The addition was made slowly, the sea water being poured down the side of the filter funnel with care. Cells were not permitted to be drawn against the surface of the filter paper during filtering; by the use of a plastic squeeze bottle of sea water they were washed away from the filter paper surface. Additional sea water was added as needed to maintain original volume. Samples of the solution passing through the filter funnel were collected and tested with the conductivity bridge against sea water at the same temperature. When the conductivities of the two solutions were found to be equal it was assumed that the medium present in the funnel was pure sea water and the transfer of cells was complete. The transferred cells were observed under the microscope for damage. If noticeable damage was present, the transfer was discarded and the process repeated. Satisfactory transfers were placed under culture conditions for six hours and periodically observed for changes in behavior. At the end of this period salinity samples were prepared as described and scanned.

It was observed that at corresponding salinities, mean velocity was consistently greater (by about 20 μ/sec.) in medium than in sea water. A two-factor analysis of variance indicated that this difference was significant ($F = 6.85^{**}$). The inter-

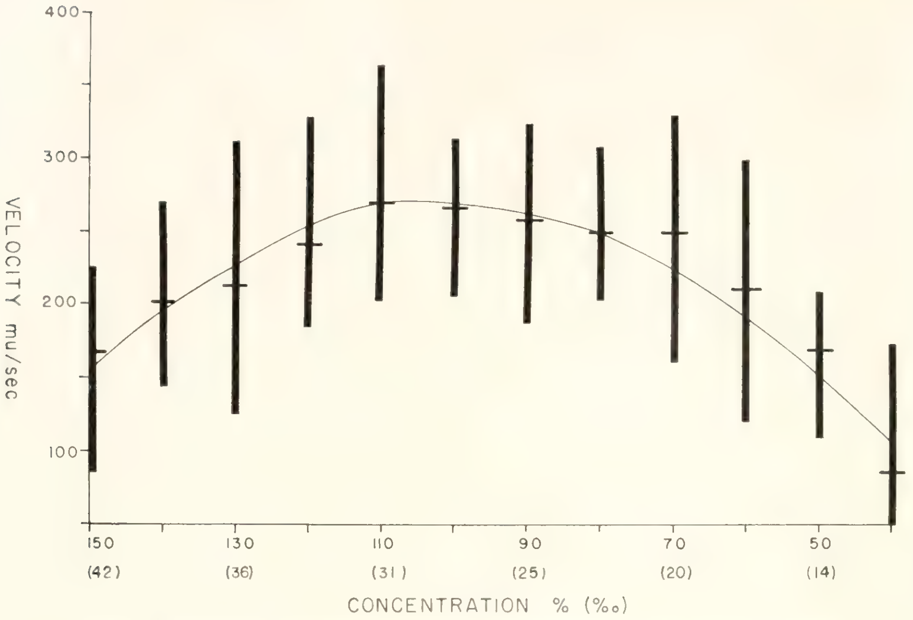

FIGURE 3. Relation of velocity to medium concentration in *Gonyaulax polyedra.*

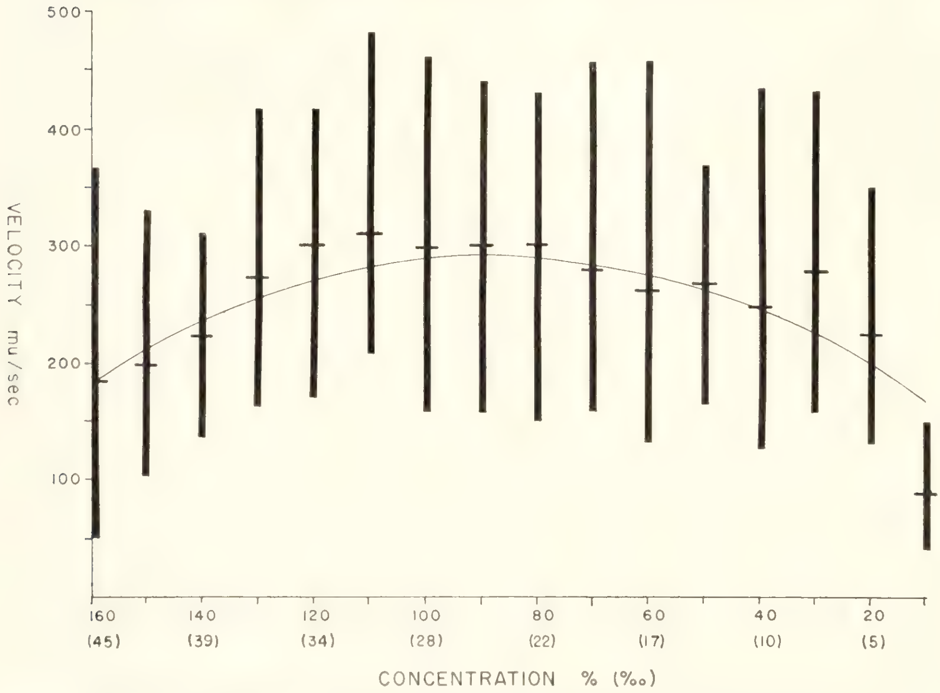

FIGURE 4. Relation of velocity to medium concentration in *Gyrodinium* sp.

action between media used and salinities tested was not significant (F = 0.96 n.s.), demonstrating that salinity change has the same effect in both media.

The "absolute" hyposalinity tolerance for motility was less in sea water than that in medium, since cells were observed to remain motile down to 50% sea-water (16 ‰), whereas in medium they retained motility down to 40% (12 ‰).

Gyrodinium

A parallel series of observations and experiments was conducted on *Gyrodinium*. As can be seen in Figure 4, *Gyrodinium* is consistently slightly faster than *Gonyaulax* over the salinity range. The graph also indicates that a plateau of velocity is maintained over a wider range of salinities than in *Gonyaulax*.

As in *Gonyaulax, Gyrodinium* has a slightly greater velocity in medium than in sea water at corresponding salinities, this difference being significant (F = 217.30***). Again, the interaction between salinity and medium used was not significant (F = 0.24 n.s.). "Absolute" hyposalinity tolerance for motility was greater in medium than in sea water, for motility was retained down to 20% sea water (6 ‰) as against 10% medium (5 ‰).

The relationship of linear velocity to temperature

Gonyaulax

As in the salinity experiments, preliminary subjective observations were conducted to determine the range in which active motility was present. The ability of cells to remain motile during a rapid temperature decline was tested by placing a culture at 20° C. in an ice-cooled water bath (8° C.). The time necessary for the culture medium to reach temperature equilibrium with the water bath was ten minutes. The cells generally maintained their motility down to a temperature of 16° C. (which took six minutes), whereupon they began to cease activity and settle to the bottom of the culture flask. All cells lost motility at 12° C. Cells kept at 8° for twelve hours did not recover motility. Cells returned to 20° immediately after the initial decline to 8° recovered activity within 12 hours.

With the use of a refrigerated chamber, the temperature of which could be lowered at a given rate and kept constant within ± 1.0° C., a combination observational and test series was conducted. The chamber was equipped with illumination of the proper intensity for culturing, this allowing a test culture to be continuously under normal light conditions. All materials necessary for the preparation of the test slides were placed in the chamber, where preparations were made as needed through a small hatch in the lid. The series was conducted by lowering the temperature in two-degree increments from 20° C. to a temperature where no motility was observed. The temperature decline between two-degree levels was achieved over a 30-minute period, this being the shortest interval possible which would not cause a subjectively observable number of cells to lose motility. At each temperature interval a sample was taken from the culture and subjected to microscopic examination. If motility was observed, test slides were made and scanned. This procedure was repeated down to a temperature of 8° C., where motility was last observed.

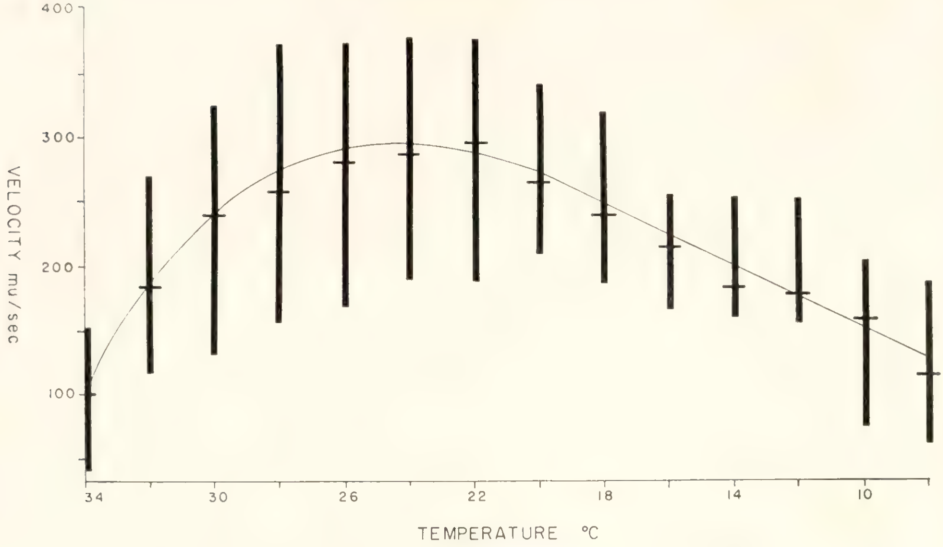

FIGURE 5. Relation of velocity to temperature in *Gonyaulax*.

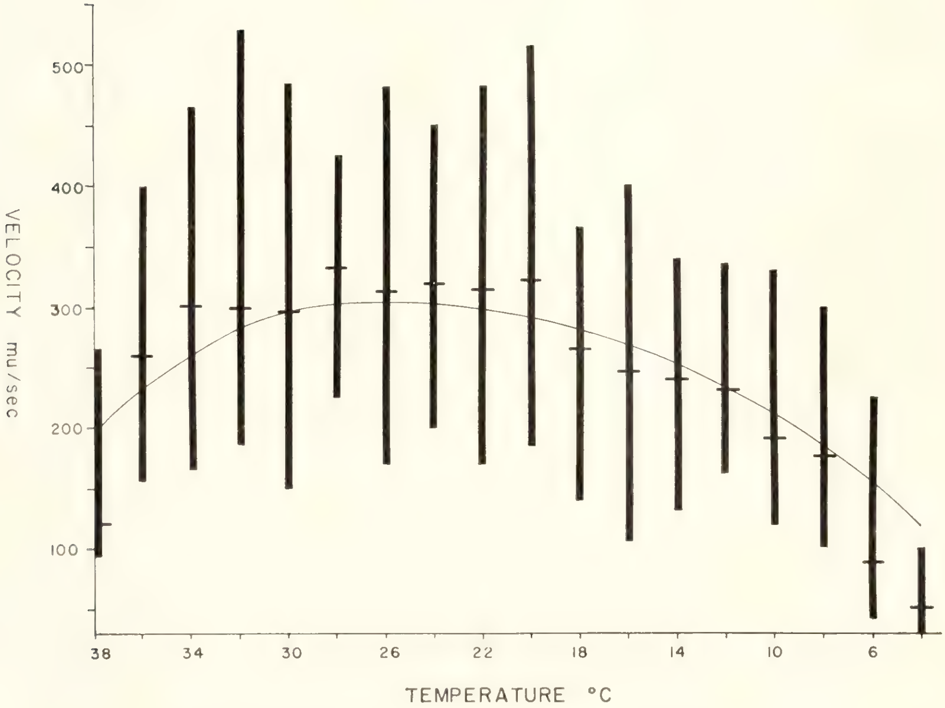

FIGURE 6. Relation of velocity to temperature in *Gyrodinium*.

The response of the cells to temperatures above 20° was subjectively determined in a manner similar to that just described. The ability of the cells to remain motile when subjected to a rapid increase in temperature was tested by placing the culture in a warm water bath which raised the temperature of the culture from 20° to 34° in approximately 20 minutes. Observations were made as before, and it was determined that the cells lost motility at 28°. Cells maintained at this temperature would recover motility in about an hour. If cells were allowed to undergo a gradual increase in temperature (approximately 2°/30 min.), they could be raised to 34° before motility ceased. Cells maintained at 34° for twelve hours never recovered motility. Cells returned immediately to 20° after being raised to 34° likewise never recovered.

The scanned and measured series was conducted by placing a single culture in a temperature-controllable water bath. This bath could maintain a given temperature within ±0.5° C. The slides used were placed in the bath in a dry, covered container. Samples were scanned at two-degree intervals from 20° C. to 34° C. Each two-degree increase was accomplished slowly over a period of not less than 30 minutes.

The relationship of linear velocity to temperature in *Gonyaulax* is shown in Figure 5.

Gyrodinium

Strictly parallel experiments with *Gyrodinium* provided the data in the curve shown in Figure 6.

DISCUSSION

As Ryther (1955) has pointed out, independent movement may be of value to dinoflagellates in competition, under conditions of changing temperature, salinity, nutrient concentration and light, particularly in forms with a phototactically-controlled diurnal migration such as *Gonyaulax polyedra* (*vide* Hasle, 1950; Halldal, 1958). We have investigated the effects of variation in the first two parameters on the swimming speed of the single cell in two species. Simple calculation reveals that the demonstrated plateau for swimming rate (Fig. 3) in *Gonyaulax* of approximately 250 μ/sec. (0.9 M./hr.) is well within the order of magnitude necessary to allow a single cell to accomplish the diurnal migration established by Hasle for *Gonyaulax* in a Norwegian fjord (approximately 10 M./12 hrs.).

The littoral *Gonyaulax* and the lagoon-inhabiting *Gyrodinium* were both found to maintain a relatively constant plateau of velocity through a wide range of salinities. *Gonyaulax* maintained a velocity plateau above 225 μ/sec. from 120% culture medium (34 ‰) through 70% culture medium (20 ‰), while *Gyrodinium* maintained a similar plateau above 250 μ/sec. from 130% culture medium (36 ‰) to 20% culture medium (5 ‰). It is clear that an adaptation of this sort to variations in salinity is a definite asset to life in estuarine and coastal waters; the wider tolerance in the lagoon-inhabiting *Gyrodinium* is to be expected. The ability of the two organisms to maintain swimming speeds within the range of their maxima means that salinity *per se* is not an important limiting factor in the distribution of cells throughout their environment, *unless* it can be shown that a change in direction or a change in rate-of-change of direction (klinokinetic response) occurs when the cell encounters a steep gradient. The former possibility cannot be investigated without a change in our technique. A brief analysis of the ratio of distance travelled over linear displacement (Surtees, 1964), to give evidence of a change in klinokinetic behavior with change in salinity, indicated no difference in the deviosity of pathways in both forms within the salinity ranges in which a constant plateau of velocity was maintained. But it must be remembered that our technique in no way parallels what might occur when a cell encounters a steep gradient, because our pathways were only recorded from cells which had had considerable time (four hours) to become adapted to the changed medium.

The apparent difference in both species in swimming speed and salinity tolerance (at low salinities) when in culture medium and when in sea water warrants brief consideration. Culture studies indicate that dinoflagellates can also reproduce normally under a considerable salinity and temperature range (Braarud, 1951; Barker, 1935) but that an essential factor for their development is the presence of certain organic chemical constituents. In our media these are supplied by soil extract. Soil extract can be supplemented or replaced by vitamin B_{12} (Provasoli and Pintner, 1953; Sweeney, 1955). In our transfer of cells from culture medium to sea water, these essential factors may have been removed from the environment, which possibly may account for the observed differences in the range of tolerance and swimming speed in our studies.

In both species velocity was similarly maintained through a wide temperature range, *Gyrodinium* again having the widest tolerance, as one might expect in a species of its habit. What is more interesting is the response of the two species to a rapid temperature decline. The encounter of such a phenomenon under natural conditions could result in marked effects on the distribution of cells in the surrounding medium, particularly in forms which undergo a marked vertical migration such as *Gonyaulax*. It is possible that cells encountering a steep drop in temperature may become inactivated and sink to a different level. They may thus become concentrated by the action of thermoclines. Many workers (Braarud, personal communication) have reported great concentrations of dinoflagellates within an area of 3–4 meters, where in the same region the non-motile diatoms were randomly distributed. J. S. Kittredge (personal communication), working at Scripps Institution of Oceanography, has shown that "microthermoclines are normally present throughout the summer in local (Southern California) near-shore waters. They vary in magnitude from a few hundredths of a degree to several tenths of a degree and, exceptionally, exceed a degree or two. Usually they are quite sharp, with a gradient of 0.1° to 1.0°/cm. depth. They vary in depth from a few centimeters to 10 M. (the greatest depth investigated). There may be several microthermoclines present in any record."

Clearly it is imperative to combine the results of such valuable microphysical oceanographic studies with observations on the behavior of the individual motile phytoplankter when it encounters a steep salinity gradient or thermocline. We know nothing about the behavior of the single cell under these conditions. We do not even know whether as a discrete and independently motile unit such a cell ever crosses an interface under natural conditions. We have not as yet directed our attention to the technical problem of "facing" the single cell with such a condition.

We believe that when we have done this and investigated the correlation of such behavior with responses effected by changes in light intensity and wave length, we may be better able to understand the so-frequently reported "spotty" distribution of these phytoplankters.

SUMMARY

1. Apparatus is described with which it is possible to make rapid measurements of linear velocity and rate of change of direction in the motile microorganism.

2. Observations on the relation between linear velocity and salinity have been made in the dinoflagellates, *Gonyaulax* and *Gyrodinium*.

3. Data are also presented on the relation of linear velocity to temperature in the two forms.

4. Results are discussed in relation to the ecology of the organisms.

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STUDIES ON HOLOTHURIAN COELOMOCYTES. II. THE ORIGIN OF COELOMOCYTES AND THE FORMATION OF BROWN BODIES

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Few accounts of the origin of holothurian coelomocytes are available. Cuénot (1897) and Hatanaka (1939) reported that coelomocytes originate in the hemal ring and vessels. Prosser and Judson (1952) mentioned the possibility that coelomocytes of *Stichopus* may originate in the hemal vessel. Hérouard (1889) indicated that coelomocytes originate in the tissues of the respiratory trees. Endean (1958) indicated that homogeneous amebocytes (lymphocytes) originated from the lining epithelium of the respiratory trees in *Holothuria leucospilota*, migrate into the coelomic fluid, and differentiate into morula cells and possibly other coelomocyte types. Hausmann (1931) claimed amebocytes were derived from the coelomic epithelium (peritoneum). Ohuye (1938) proposed that the category of coelomocytes known as lymphocytes (see Hetzel, 1963) represented primitive pluripotent cells from which all other coelomocyte types are derived. Théel (1920) indicated crystal coelomocytes were derived from amebocytes.

No reports describing the formation of holothurian brown bodies are known. The present study is an attempt, first, to clarify the origin of coelomocytes and their patterns of differentiation, and second, to provide some knowledge of the formation of brown bodies in holothurians.

MATERIALS AND METHODS

In hope of increasing the rate of coelomocyte production, fifteen specimens of each of the following four species of holothurians were bled daily for five consecutive days: *Cucumaria miniata*, *Eupentacta quinquesemita*, *Parastichopus californicus*, and *Psolus chitonoides*. Perivisceral coelomic fluid was withdrawn with a hypodermic syringe and needle. As much as 3 cc. of coelomic fluid could be withdrawn from a large *Cucumaria* during the first bleeding, but increasingly smaller amounts of coelomic fluid were obtainable during subsequent bleedings. Bleeding did not cause evisceration in the species studied. Following the series of bleedings, the holothurians remained undisturbed for two days. On the eighth day, samples of coelomic fluid were withdrawn from experimental animals and from control animals that had not been previously bled. Smears of this coelomic fluid were vitally stained with toluidine blue and Janus green B, as described by Hetzel (1963). Other smears were fixed with osmic acid or formalin vapors for 30 to 60 minutes, air-dried, and stained in a variety of ways. The periodic acid-Schiff

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(PAS) technique, the Alcian blue method, and the ribonucleic acid extraction technique, as outlined in Pearse (1960) on page 832, 838, and 916, respectively, were used. Some smears were stained with the mercuric chloride bromphenol blue technique of Mazia, Brewster and Alfert (1953), or with hematoxylin and eosin.

Both experimental and control animals were sacrificed on the eighth day. Samples of the body wall and all major organs were fixed with Bouin's fixative, dehydrated in ethyl alcohol, embedded in paraffin, and sectioned at 10 microns. Sections were stained with hematoxylin and eosin. All sections and smears were searched for sites of coelomocyte production.

Brown body formation was studied by means of injections of a 2% (W/V) suspension of lamp black (carbon) in filtered sea water into the perivisceral coelom of holothurians. Thirty specimens of *Cucumaria miniata* were injected with 2 to 4 ml. of the lamp black suspension, depending on the size of the organism; five specimens of *Eupentacta quinquesemita* and five of *Psolus chitonoides* were each injected with 1 ml. of the suspension. Each of five specimens of *Leptosynapta clarki* was injected with 0.5 ml. The injected animals were placed in individual fingerbowls or aquaria filled with sea water. One-ml. samples of coelomic fluid were withdrawn for examination after 30 minutes, 60 minutes, 2, 3, and 8 hours, and, thereafter, once daily for 30 days.

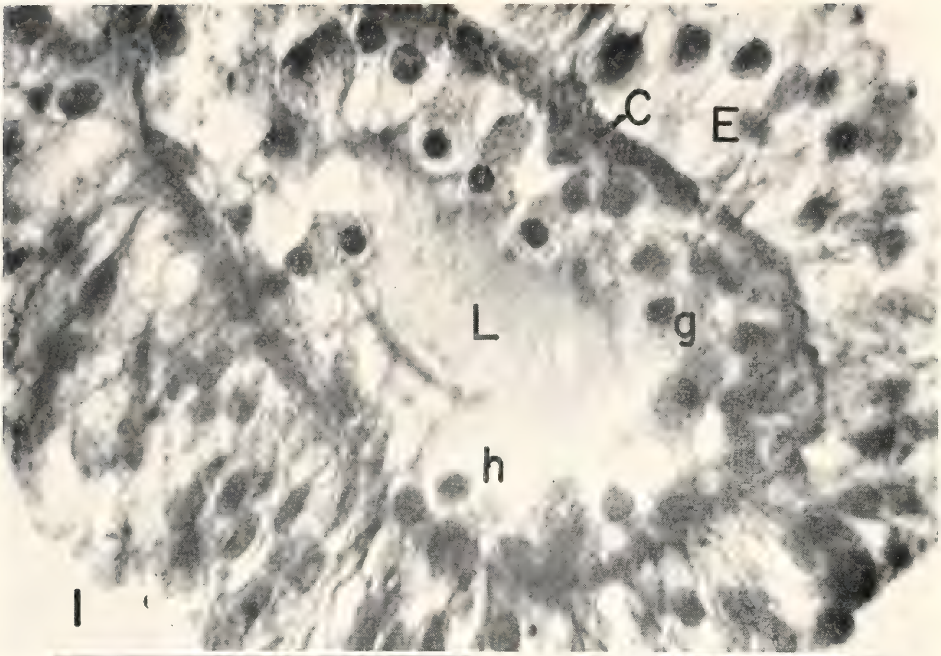
One *Cucumaria* was sacrificed every other day for a period of one month and after that at irregular intervals for up to six months. One *Psolus* was sacrificed each week for four weeks. The animals were dissected and searched for sites of carbon deposition. The entire digestive systems and respiratory trees were removed, and bleached and hardened in 95% ethyl alcohol for 12 hours before they were dissected and their contents and walls examined for the presence of black carbon deposits. No specimens of *Eupentacta* or *Leptosynapta* were sacrificed during the course of the experiment.

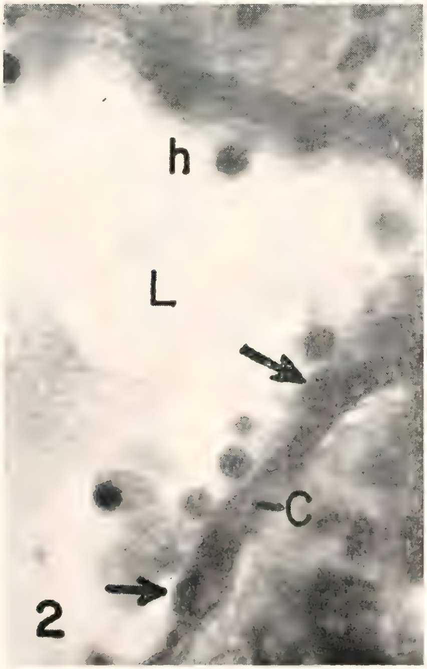
Results

No coelomocyte free in the coelomic fluid of experimental or control animals was ever observed in any stage of cell division. Samples of coelomic fluid taken from experimental animals showed no obvious differences from samples taken from control animals. Among the coelomocytes present in the samples taken from control and experimental animals were cells which could be arranged in graded series between lymphocytes and hemocytes, and between lymphocytes and amebocytes.

I. Series of cells intergrading between lymphocytes and hemocytes.

1. Larger than average oval to spherical lymphocytes, with an increased number of Janus green B-positive granules.
2. Cells as described above with one or more of the Janus green B-positive granules containing a clear vacuole.
3. Still larger lymphocyte-like cells, similar to those of type 2 above, but with hyaline cytoplasm tinged straw-yellow.
4. Cells slightly smaller than hemocytes and with straw-yellow cytoplasm, but without the typical yellow refractile granules of hemocytes.





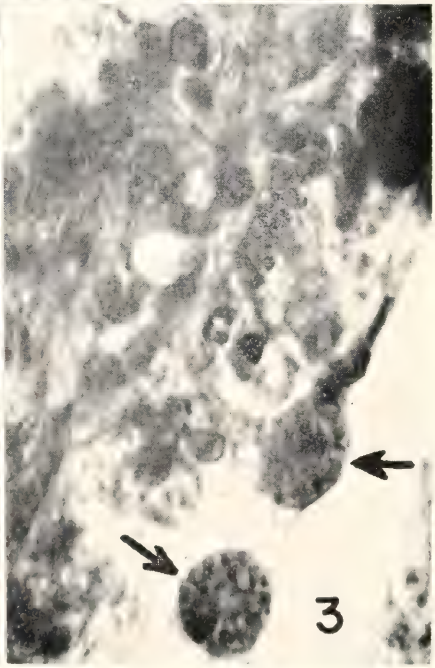


FIGURE 1. Cross-section of the dorsal hemal vessel of *Psolus chitonoides* (970 ×). C, connective tissue layer; E, external epithelium; L, lumen of vessel; g, lymphocyte with granular cytoplasm; h, lymphocyte with hyaline cytoplasm.

Cells belonging to Series I were observed in *Cucumaria* and *Eupentacta,* but not in *Parastichopus* or *Psolus.*

II. Series of cells intergrading between lymphocytes and amebocytes

1. Unusually large lymphocytes with more than three filiform pseudopodia.
2. Larger cells with still more complicated pseudopodial systems and increased granulation.
3. Cells that approach amebocytes both in size and in degree of cytoplasmic granulation.

Cells belonging to Series II were found in all four species of holothurians examined.

No histological differences were noted between tissues taken from experimental animals and tissues taken from control animals. I found no evidence of the origin of coelomocytes from the peritoneum covering the inner surfaces of the longitudinal retractor muscles. No observations suggested that coelomocytes originate from the walls of the respiratory trees, nor from the body wall, the wall of the digestive tract proper, the polian vesicles, the water vascular system in general, nor from any part of the reproductive system.

Sections of the hemal vessels showed numerous lymphocytes within the lumina of the vessels (Fig. 1). The connective tissue layers of the hemal vessels and their associated mesenteries contained numerous mesenchymal cells with flattened, intensely staining nuclei. Mesenchymal cells located nearer the lumen of the hemal vessels contained oval, less intensely staining nuclei. Lymphocyte-like cells with oval nuclei and hyaline cytoplasm bulged from the connective tissue into the lumen of the hemal vessels (Fig. 2). Many of the latter cells formed a series exhibiting increasing size and granulation. These granulated cells can be arranged in a series intergrading with basophilic morula-like cells contained in the connective tissue layer of the mesenteries associated with the hemal vessels and the digestive tract (Fig. 3).

III. Series of cells intergrading between lymphocytes in the hemal vessels and basophilic morula-like cells.

1. Enlarged lymphocytes with homogeneous eosinophilic cytoplasm.
2. Enlarged cells as above, with finely granular eosinophilic cytoplasm; these cells were also observed in the connective tissue of the mesenteries associated with the hemal vessels.
3. Cells with amphiphilic staining properties and with granular cytoplasm located in the mesenteric connective tissue.
4. Amphiphilic cells in the connective tissue of the mesenteries with morphological features identical to those of morula cells.
5. Basophilic morula cells contained in the connective tissue layer of the mesenteries and free in the coelomic fluid.

FIGURE 2. Cross-section of hemal vessel of *Psolus chitonoides* (1292 ×). Arrows indicate oval nuclei of mesenchymal cells believed to be differentiating into lymphocytes. C, connective tissue layer; L, lumen of vessel; h, lymphocyte with hyaline cytoplasm.

FIGURE 3. Section of dorsal mesentery of *Psolus chitonoides* (970 ×). Arrows indicate basophilic morula cells.

Numerous eosinophilic morula cells were present throughout the connective tissues of the body other than the connective tissues of the gut, hemal vessels, and mesenteries adjacent to the hemal vessels and gut.

Spherules of morula cells fixed and stained at the moment of their release from cytolyzing cells were composed of an outer basophilic and metachromatic shell, and an inner core which was eosinophilic, but not metachromatic. The outer shell was PAS-positive and stained green with Alcian blue; the inner core stained blue when exposed to Mazia's bromphenol blue technique. Morula cells lost their basophilia following treatment with ribonuclease.

The injection of lamp black suspension into the coelomic cavity of holothurians apparently had little effect on the animals. Holothurians left undisturbed following the injection were observed to feed normally after one hour. Injected specimens were still normal and healthy six months after the injection.

Samples of the coelomic fluid withdrawn 30 minutes following the injection contained very few coelomocytes when compared to the number normally present. A sample taken one hour after the injection contained many more cells than did the previous sample, but the number was still clearly below normal. A few petaloid amebocytes contained engulfed carbon particles at this time, but these represented only a small per cent of the amebocytes present in the sample. In *Cucumaria,* carbon particles were occasionally observed within hemocytes. In many instances several amebocytes were observed clustered around masses of carbon particles.

The number of amebocytes containing engulfed carbon particles gradually increased up to the tenth to the fourteenth day following injection. By that time, almost every amebocyte contained carbon particles. Lymphocytes, morula cells, and crystal cells were never observed containing engulfed carbon.

Between the third and fourth weeks following injection the number of amebocytes containing carbon particles gradually decreased, and the number of amebocytes free of carbon particles increased. After six months an occasional amebocyte still contained particles of engulfed carbon. Amebocytes heavily laden with engulfed carbon particles did not produce large networks of filiform pseudopodia and, therefore, did not contribute actively to the formation of clots as did amebocytes free of carbon.

Most amebocytes with engulfed carbon particles also contained yellow granular material similar to the granular material composing brown bodies. Many spherical cells, completely filled with yellow granules and/or carbon particles, were observed.

Large masses of carbon were still found in the perivisceral coelomic cavity of *Cucumaria* after six months. As early as two days following the injection, noticeable accumulations of carbon were observed in the anterior portions of the water vascular system, especially in the polian vesicle, and in the ampullae of the anterior tube feet. Large masses of carbon were also located among the suspensor muscles of the cloaca, and along the junction of the dorsal mesentery and the gut. Carbon was accumulated in the same locations in *Psolus chitonoides.* After two weeks, carbon was also noted among the deposits of yellow granular material between the peritoneum and the wall of the sole of *Psolus.*

The walls of the excised gut and respiratory trees of *Cucumaria* and *Psolus* were searched for carbon deposits. Specks of carbon were occasionally found in the anterior and mid-gut regions two days following the injection, but no carbon was detected in the lumina or walls of the respiratory trees. Occasionally, small amounts of carbon were found in the feces of *Cucumaria* and *Eupentacta*. After the second day, brown bodies composed partly of carbon were found in the aquaria containing injected *Cucumaria* and *Eupentacta*. Masses of amebocytes laden with carbon emerged from the bases of the tube feet of *Cucumaria*. Several specimens of *Cucumaria* were covered with carbon-laden mucus. After a period of three weeks the noticeable emission of carbon from the holothurians decreased and finally stopped.

Following the injection of lamp black, the normally clear coelomic fluid visible through the body wall of *Leptosynapta* appeared cloudy. Within two hours, however, the coelomic fluid was again clear and the carbon was concentrated in the ciliated funnels that line the junction of the mesenteries and the body wall of the animal.

The elimination of the carbon deposits from the ciliated urns of *Leptosynapta* occurred more rapidly than the elimination of carbon from bodies of the other holothurians studied. Within three days most of the carbon was gone from the ciliated funnels of *Leptosynapta*. Masses of carbon-laden amebocytes migrated from the ciliated urns through the body wall of *Leptosynapta* to the exterior.

DISCUSSION

On the basis of observations made in the present study, one is led to support Ohuye's (1938) conclusion that the various coelomocyte types are possibly derived from a common source or stock of stem cells, and that all types of coelomocytes may be produced by direct transformation of the stem cells. Lymphocytes are probably the stem cells which differentiate into hemocytes, amebocytes, and morula cells. The source of crystal-bearing coelomocytes is unknown at the present time, but Théel (1920) believed that they differentiated from amebocytes.

The cells in Series I may be interpreted as lymphocytes undergoing differentiation into hemocytes. This view is further supported by the finding that these intermediate stages are found in *Cucumaria* and *Eupentacta* which possess hemocytes, but not in *Parastichopus* and *Psolus* which lack hemocytes (Hetzel, 1963). The course of differentiation is presumed to be the following: lymphocytes free in the coelomic fluid enlarge and produce increased numbers of Janus green-stainable granules; as hemoglobin synthesis occurs, the cytoplasm becomes straw-yellow in color and the large yellow refractile granules typical of holothurian hemocytes appear; the refractile granules appear to be derived from the Janus green-positive cytoplasmic granules.

Cells in Series II may be interpreted as lymphocytes differentiating into amebocytes. Lymphocytes are presumed to increase in size and produce more complex systems of pseudopodia as they differentiate into amebocytes.

No evidence was found in the present study to indicate that morula cells differentiated from lymphocytes free in the coelomic fluid as Endean (1958) proposed. Observations indicate that lymphocytes in the hemal vessels and closely associated gut mesenteries may form morula cells. The course of this differentiation is believed to be as follows: the hyaline cytoplasm of these lymphocytes gradually becomes granular; the granules gradually increase in size and are transformed into the spherules typical of morula cells; during the transformation of the granules into spherules, the cytoplasm of the lymphocytes changes from eosinophilic to amphiphilic to the basophilic condition typical of morula cells located in the connective tissue framework of the mesenteries and morula cells free in the coelomic fluid.

The basophilia of morula cells was removed by ribonuclease digestion, and the cores of the spherules of the morula cells appear to be protein, as indicated by their positive staining reaction to Mazia's bromphenol blue technique. These observations indicate that morula cells may play an important role in amino acid uptake from the digestive tract and in protein synthesis and storage. If the morula cells indeed do function in amino acid uptake and in protein synthesis, as available evidence suggests, their possible site of origin in the hemal vessels, which are so closely related to the digestive tract, would be especially significant. Eosinophilic morula cells found throughout the connective tissue layers of holothurians possibly represent morula cells that have completed their synthetic activities and await an as yet undescribed function.

Endean (1958) reported that spherules of morula cells of *Holothuria leucospilota* also consisted of an inner proteinaceous core and an outer metachromatic shell believed to be, at least in part, mucopolysaccharide. The present study confirms his observations of the structure of the spherules of morula cells, and supports Endean's theory that they differentiate from lymphocytes. In species studied in the present report, however, morula cells appear to differentiate in close association with the hemal vessels, and not in the perivisceral coelom, as Endean described.

None of the observations made in the present study contribute to an understanding of the formation of crystal cells in holothurians. The crystals are not able to withstand the effects of fixation and preparation of tissue sections. Possible stages in the formation of crystal cells were likely destroyed in the prepared tissues, and only fully differentiated crystal cells were observed in living material. Théel (1920) claimed that crystal cells were derived from ameboid cells.

The fact that crystal cells, hemocytes, and morula cells are able to produce pseudopodia of some form (Hetzel, 1963) lends credibility to the hypothesis that these cells may all be derived from a common form that also gives rise to amebocytes.

The site of origin of lymphocytes proved to be a problem not easily resolved. Hausmann (1931) maintained they originate from cells of the peritoneal lining of the coelom. This may be so, but would be difficult to prove conclusively. Ohuye (1938) does not rule out the possibility that mesenchymal connective tissue cells may contribute to the formation of lymphocytes in holothurians.

Observations made in the present study indicate that at least some of the lymphocytes may differentiate from mesenchymal connective tissue cells in the walls of the hemal vessels. I believe that lymphocytes more likely are derived from mesenchyme than from epithelial cells such as those that make up the peritoneum. My observations suggest that lymphocyte-like cells in the hemal vessels possibly differentiate into morula cells. Other lymphocytes conceivably could migrate from their site of origin into the coelomic cavities, and there differentiate into hemocytes and amebocytes.

Schinke (1950) studied the origin and differentiation of coelomocytes of the echinoid, *Psammechinus miliaris*. Schinke reported that white amebocytes (lymphocytes?) were formed by direct transformation of cells in the connective tissue matrix of the calcareous tests. Schinke stated that the formation of the coelomocytes never involved mitosis, nor did the coelomocytes themselves ever undergo cell division. Holothurian coelomocytes have not been observed in cell division.

In light of Schinke's study, and the observations made in the present investigation, it seems reasonable to assume that lymphocytes are produced from mesenchymal connective tissue. The observations of Hatanaka (1939) of cells in the hemal vessels of holothurians also support this hypothesis.

Cuénot (1897) defined a lymphoid organ in invertebrate animals as an organ closely related to the blood cells and coelomocytes, and one that is composed of amoeboid cells or produces them. Cuénot described a lymphoid organ as a connective tissue framework in which are found many corpuscles identical to those that occur free in the body fluid. On the basis of Cuénot's definition, and on suggestions proposed in the present study, the hemal system of holothurians may be regarded as lymphoid tissue that functions in part to produce cells from which the coelomocytes are derived. Cuénot, himself, considered the hemal ring of holothurians as a lymphoid organ. The rete mirabile, an extension of the hemal system, may also function in part in the formation of coelomocytes.

On the basis of a survey of the coelomocytes of various invertebrate animals, including two species of holothurians, Ohuye (1938) derived lymphocytes from mesenchymal and/or peritoneal cells. Ohuye derived erythrocytes (hemocytes), amphiphilic, eosinophilic, and basophilic granulocytes (morula cells), and pigmented leucocytes from lymphocytes.

The existence of holothurian coelomocytes possessing characteristics intermediate between lymphocytes and hemocytes, morula cells, and amebocytes, lends support to Ohuye's views of coelomocyte lineages in holothurians.

Amebocytes appear to have at least two functions in holothurians, as phagocytes and as active agents in the clotting reaction. These functions appear to compete against one another; two weeks after the injection of carbon into *Cucumaria,* when the amebocytes were at peak activity in disposing of injected carbon, the efficiency of the clotting mechanism was low. Later, when more amebocytes were again free from the chore of carbon elimination, the efficiency of the clotting reaction returned.

The inability of carbon-laden amebocytes actively to contribute to the formation of a clot may easily be explained. As the amebocytes became filled with carbon particles, they became less ameboid and were eventually transformed into spherical cells laden with carbon; some also contained yellow granular material. Amebocytes partially filled with carbon granules were impaired in their ability to produce the extensive networks of filiform pseudopodia necessary for clot formation.

Observations of hemocytes containing small quantities of engulfed carbon were especially interesting. This is the first report of such an observation. The weakly phagocytic nature of hemocytes could indicate some relationship between hemocytes and amebocytes and lends further support to Ohuye's (1938) scheme of coelomocyte lineages.

The inclusion of carbon within brown bodies supports the hypothesis that brown body material must be considered a product, if not truly excretory, at least of no use to the holothurian. The fact that carbon is engulfed by amebocytes that often contain yellow granules, and the fact that the carbon-laden cells are accumulated in the same region of the body where masses of yellow granules were found, would seem to indicate that the injected carbon particles and yellow granular material are both disposed of by phagocytic amebocytes. The additional fact that yellow granules and carbon appear together in brown bodies would also indicate that brown bodies are indeed a material that is actively accumulated by amebocytes and eliminated by the holothurian. The presence of brown bodies in holothurians which both possess and lack hemocytes would seem to indicate that these granules are not necessarily related to hemoglobin synthesis or degradation.

The lack of carbon in any of the morula cells or crystal cells would seem to indicate that these cells are very specialized and have lost all powers of phagocytosis.

One other speculation may be made regarding the phagocytic nature of amebocytes and their role in removing unwanted materials from the body of holothurians. Hetzel (1963) reported amebocytes and brown bodies containing one or more hemocytes, morula cells, or crystal cells. These observations suggest that the elimination of old or damaged coelomocytes from the bodies of holothurians might be performed in a manner similar to the elimination of carbon or yellow granular material. If this were true, the constant loss or elimination of these coelomocytes in brown body material would require the continued replacement of these coelomocytes and, therefore, the continued production of new coelomocytes throughout the life of the holothurian.

SUMMARY

1. Coelomocyte and brown body production were studied in four species of holothurians, *Cucumaria miniata*, *Eupentacta quinquesemita*, *Parastichopus californicus*, and *Psolus chitonoides*.

2. Available evidence suggests that at least some, if not all, lymphocytes possibly originate from mesenchymal cells in the hemal vessels of holothurians, and possibly later differentiate directly into hemocytes, amebocytes, and morula cells.

3. The coelomic fluid of *Cucumaria* and *Eupentacta* contains coelomocytes which can be arranged in a series intergrading between lymphocytes and hemocytes. These cells possibly represent stages in the transformation of lymphocytes into hemocytes.

4. *Parastichopus* and *Psolus,* which lack hemocytes, do not possess the series of cells interpreted as stages in hemocyte differentiation.

5. All four species of holothurians studied possess coelomocytes which can be arranged in a series intergrading between lymphocytes and amebocytes. These cells possibly represent stages in the differentiation of lymphocytes into amebocytes.

6. Granular lymphocyte-like cells located in the hemal vessels of the four species studied possibly represent stages in the differentiation of lymphocytes into morula cells.

7. *Cucumaria miniata*, *Eupentacta quinquesemita*, *Psolus chitonoides*, and *Leptosynapta clarki* were injected with suspensions of lamp black in sea water.

8. The carbon particles were engulfed by amebocytes which often also contained granules similar to the granules found in brown bodies.

9. Carbon particles were accumulated in the same regions of the body as brown body material.

10. Brown bodies composed partly of carbon particles were eliminated by the holothurians.

11. Amebocytes are believed to play a role in the accumulation of brown body material and in the production of brown bodies.

12. Carbon particles were also concentrated in the ciliated funnels or urns of *Leptosynapta clarki*.

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DESCRIPTION OF HEMOCYTES AND THE COAGULATION PROCESS IN THE BOLL WEEVIL, ANTHONOMUS GRANDIS BOHEMAN (CURCULIONIDAE)[1]

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This report presents the results of a study of the hemocytes of the boll weevil, *Anthonomus grandis* Boheman. A knowledge of the hemocytes of healthy boll weevils was considered necessary before detailed study of the effect of various pathological conditions upon hemocytes could be conducted. Wittig (1962), in reviewing the pathology of insect blood cells, observed that diseases may affect hemocytes directly or indirectly. Knowledge of these effects may be useful not only in studying a microbial disease which attacks the hemocytes, but also as an aid to bioassaying a population for pathological conditions. Knowledge of the hemocytes of healthy insects is necessary for such comparisons.

TERMINOLOGY

Classification of hemocytes provides a means of reference for further studies concerning their function, changes due to normal physiological processes or to abnormal conditions, and for comparison with hemocytes in other species. Classification systems have classically depended upon morphological characters. As Wigglesworth (1959) stated, determination of function should be most important. Nevertheless, recognition of hemocyte morphology is necessary before detailed functional studies can be conducted. Classification on a purely functional basis would result in much confusion, since a distinct cell type often has more than one function. Moreover, hemocytes of dissimilar morphology from different species have similar functions. Accordingly, classification of boll weevil hemocytes has been established on the basis of morphological similarities, following to a large extent the system used by Jones (1962). When a distinct hemocyte type was observed to transform *in vitro*, the variations in form were described; but these were not considered to constitute a distinct type of hemocyte, especially since *in vitro* changes may not have been a true representation of *in vivo* activity. This factor was further emphasized by the conditions which created artifacts during the investigation.

The types of hemocytes present in the boll weevil correspond to previously recognized hemocyte groups of other insects. We feel that this system of nomenclature may serve as a basis for common usage, by insect pathologists at least,

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until further data regarding origin and function warrant a change. Each insect species apparently has its own characteristic hemocyte picture. Basic hemocyte types exist, but morphological adaptations to varying physiological requirements and conditions occur. The reader is referred to the excellent reviews by Wigglesworth (1959) and Jones (1962) for a more extensive coverage. The literature which served as a basis for classification is reviewed briefly in the following paragraphs to indicate the reasons for usage of the terms and to relate the terms to those which have been considered synonyms.

Prohemocytes: Prohemocytes are generally characterized by having a small amount of cytoplasm, the nucleus comprising the greater amount of the cell volume. These cells develop into other types which presumably are then capable of performing functions of mature hemocytes. The term "prohemocytes" was used by Jones (1950) and Arnold (1952). Jones (1959) stated that these are the same as Yeager's (1945) proleucocytes. The term "prohemocyte" is proposed as the standard.

Plasmatocytes: Hemocytes usually characterized by their pleomorphic capability and varied functions, as well as being the most abundant in the host, have been termed "plasmatocyte" by Yeager (1945). Yeager recognized a large number of cell types which differed mainly in their morphology. Arnold (1952) used the term in a report in which he included many of Yeager's types under that term. Jones (1959) retained Yeager's classification of plasmatocyte but also retained "podocytes" and "vermiform cells." Jones (personal communication) reported that podocytes or vermiform cells had not been observed to alter their form *in vitro*. The term "plasmatocyte" was adopted in this study to designate the hemocytes which were pleomorphic, exhibited cytoplasmic variations, were capable of altering their shape *in vitro* and were obtained in corresponding shapes in fresh preparations of blood, were capable of phagocytosis and took part in a coagulation process. Until further study can determine separate, distinct cell types for these functions, it is proposed that this term be used to designate such hemocytes.

Adipohemocytes: Hemocytes with lipoid or other refractive inclusions were termed "spheroidocytes" by Yeager (1945) and Jones (1959). Later, Jones (1962) preferred the term "adipohemocytes," since "spheroidocytes" could be confused with a distinctly different type—"spherule cells." The term "adipohemocyte" is proposed as the standard. The reader is referred to this review for further discussion of adipohemocytes.

Spherule cells: Yeager (1945) described "eruptive cells," which Jones (1959) regarded as "spherule cells." Jones (1962) further reviewed the usage of this term. These cells contain one to several large, distinct, non-refringent, amorphous inclusions which often distend the cell membrane. These inclusions are often expelled into the hemolymph *in vitro* but are not associated with coagulation. They are a distinct and easily recognized type of cell. The term "spherule cell" is preferred because it adequately describes the distinctive appearance of the cell and does not relate to an activity which may or may not be an artifact, but which certainly can be affected by conditions attendant to the technique employed to study the cells.

Oenocytoids: Yeager (1945) described oenocyte-like cells free in the hemolymph. Arnold (1952) used the term oenocytoids. These have a small nucleus, a compact cytoplasm which is usually opaque, are lightly basophilic or eosinophilic and frequently contain a small number of granules or crystals. The cell membrane may be lightly creased, have elaborate canaliculi, or be complexly folded (Hollande, 1911; Jones, 1959; Yeager, 1945). Oenocytoids are distinct from the non-circulating specialized cells known as oenocytes which are enormous acidophilic cells of ectodermal origin, usually segmentally arranged in the abdomen. The term "oenocytoids" is preferred to "oenocyte-like" cells for these reasons.

Materials and Methods

Larvae were used for the initial descriptive work and an attempt was made to select those of uniform size and physiological condition to eliminate possible variations due to growth or developmental stages. Later, hemocytes were examined from all larval stages and from adults. Larvae were utilized soon after collection from local cottonfields, to reduce artifacts which might have resulted from abnormal laboratory conditions for the insects. Larvae were placed ventral side up in modelling clay and fastened securely. A sealed #30 hypodermic needle was used to make a puncture at the midventral line, which was free of adipose tissue formation.

The primary method of observation was of wet mounts with a Leitz Ortholux[4] microscope by phase contrast, bright-field or ultraviolet illumination. A Metrimpex (Budapest) 3-D Condenser[4] (National Instrument Laboratories, Inc., 12300 Parklawn Drive, Rockville, Maryland, U. S. A.) was also used. Fixed and stained preparations were utilized for histochemical and comparative purposes.

Two methods were used for obtaining hemolymph and placing it on a coverslip. The direct method consisted of touching a coverslip to the exuding hemolymph. The capillary method involved collection in a capillary tube made from a 1-mm. capillary tube finely drawn and fire-polished. The hemolymph was expelled onto a coverslip. Approximately 1 to 3 μl. of hemolymph were easily obtained from larvae.

The coverslip was then inverted over a slide and supported by a thin ring of white petroleum jelly so that a deep layer of hemolymph, not compressed by the weight of the coverslip, was formed. A similar preparation was obtained by use of a Leitz culture trough slide (PHOLD).[4] Drying of the hemolymph was negligible and often a preparation remained satisfactory for 12 to 17 hours.

Some preparations were diluted about 1:1. Dilution of hemolymph was accomplished prior to placing the coverslip over the slide. The diluting solutions were: water, pH 7.3; 0.85% NaCl solution; 1.0% potassium oxalate solution; distilled water, pH 6.1; 0.5% trehalose solution in distilled water, final pH 5.8; phosphate buffer, pH 6.4. Fluorescent dyes were also added. Acridine orange, auramine 0, and rhodamine B were prepared at 1×10^{-3} concentration in phosphate buffers of pH 6.4 and 7.2. The phosphate buffers were prepared by mixing the proper amounts of 0.07 M Na_2HPO_4 and KH_2PO_4 solutions. No autofluorescence occurred.

Stained preparations were made by rapidly air-drying the fresh, unfixed hemolymph. Some preparations were then subjected to 40% formaldehyde or absolute methanol fixation for several minutes. The stains used were Sudan IV, saturated iodine (Lugol's solution), Delafield's or Heidenhain's iron hematoxylin, fast green FCF, Giemsa, and Wright's blood stain.

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[4] The use of trade or proprietary names does not necessarily imply the endorsement of these products by the U. S. Department of Agriculture.

Hemocyte Description

Prohemocytes. These were disc-shaped, pale gray, and nearly homogeneous by phase contrast. The nucleus occupied nearly the entire cell and only a thin band of peripheral cytoplasm was present. Cells averaged 7.0 μ ±1.3 μ, range (R) 6.3–9.9 μ, number (N) = 11 (Plate I, Fig. 1). When stained with acridine orange, the large nucleus and small band of cytoplasm were very prominent. These cells were relatively rare and only in young first instars were they fairly numerous.

Variations of this cell were observed which formed a complete range of developmental stages. The cytoplasm increased in quantity and progressively became more optically dense to phase contrast. A small number of granular inclusions were often present and large granules or vacuoles were occasionally seen in cells near the end of maturation. The final product was indistinguishable from disc-shaped plasmatocytes.

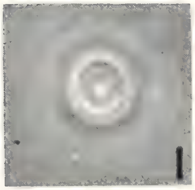
Plasmatocytes. Plasmatocytes were characterized mainly by their morphological variability. The cytoplasm was finely granular, dense, and uniform; or it contained various larger (about 1 μ) dark granules, exhibited areas of different density, and displayed small vacuoles or inclusions which were either gray or highly refractive by phase contrast. The nucleus was usually quite large and finely punctate or granulated. Plasmatocytes can become hyaline or highly refractive at their periphery. Disc-shaped plasmatocytes (Plate I, Fig. 2) measured 9.7 μ ±1.9 μ (R = 6.4–15.3 μ, N = 75). The same data showed nuclei to measure 5.3 μ ±1.05 μ (R = 3.4–8.5 μ).

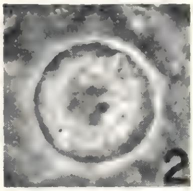
A common variant form of plasmatocyte is shown in Plate I, Figures 5 and 6. These cells had a granular cytoplasm and sometimes contained small vacuoles, granular inclusions, or dark, gray, small inclusions. They were also seen with one or two highly refractile lipoid globules. These were termed adipoid plasmatocytes.

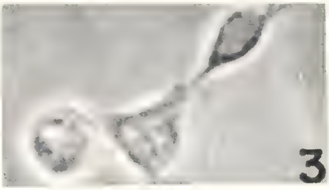
The frequency of cells with these inclusions was higher when free lipoid droplets were present in the hemolymph. Intentional disruption of adipose tissue, by rolling the larvae with pressure or by use of a needle to disrupt the tissue, produced a similar effect. The plasmatocytes were never observed ingesting lipoid droplets. The natural occurrence of free lipoid droplets was observed during the prepupal stage of larval development.

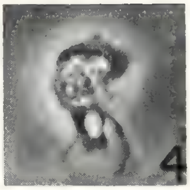
Another form was characterized by possession of one to several hyaloplasmic extensions (Plate I, Figs. 4 and 5). When movement of the fluid caused the cells to be carried along, they would often temporarily attach to the surface by these extensions. This form was observed to change into a disc-like shape or to attach at one point and elongate into a fusiform, or spindle-shaped, cell. This type was highly unstable *in vitro*.

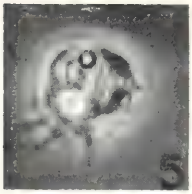
The fusiform cell (Plate I, Fig. 3) was also seen upon immediate examination of a preparation, but was not as frequent as disc-shaped cells or cells with cytoplasmic extensions. The fusiform cell could also be formed directly from a disc-

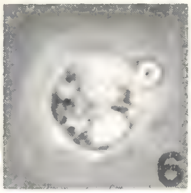


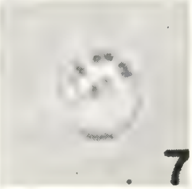


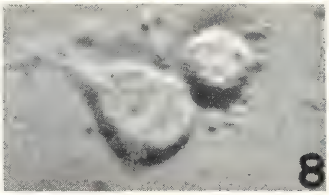


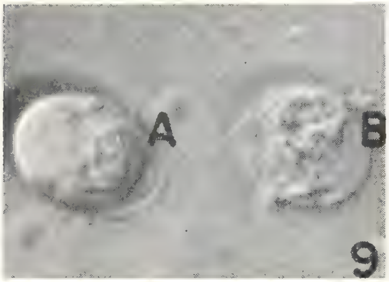


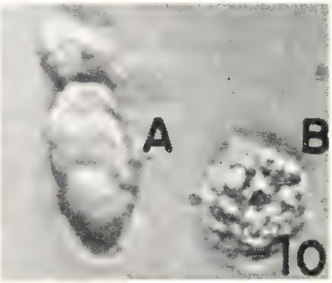


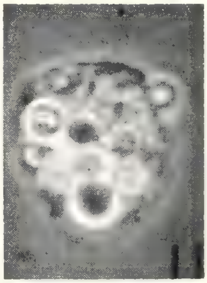


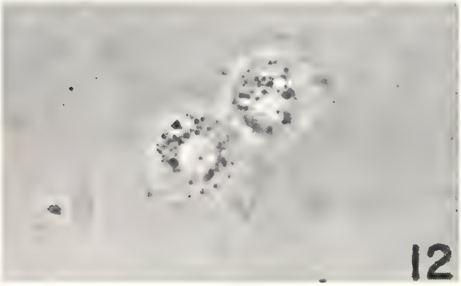


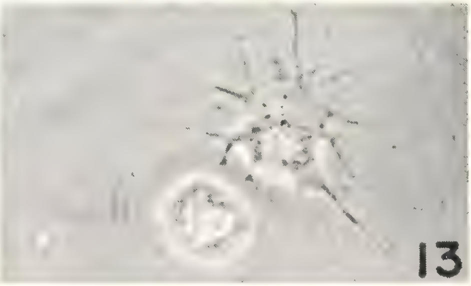


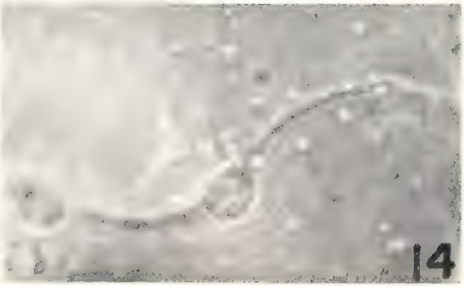


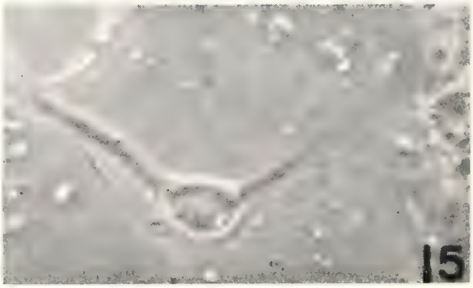












All preparations were of fresh, unfixed, undiluted hemolymph.

Plate 1

FIGURE 1. Prohemocyte. Phase contrast. 875 ×.

FIGURE 2. Disc-shaped plasmatocyte. Phase contrast. 1090 ×.

shaped plasmatocyte. A teardrop-shaped cell was also formed from disc-shaped plasmatocytes by elongation after attachment to the glass at the other end of the cell (Plate I, Fig. 8). The cytoplasm varied from dense and uniform but finely granulated to a differentiated cytoplasmic area of slight refractivity by phase contrast and containing small vacuoles, or dark granules. Extreme elongation resulted in a thinner cell in which these contents were more easily observed. The spindle-shaped cell often had tenuous, hyaline, filiform, cytoplasmic extensions from either end which exhibited a searching and probing motion. This activity was fairly slow and not whiplike. The incidence of these cells increased as the preparation remained undisturbed on the microscope stage.

Disc-shaped plasmatocytes were also capable of transformation into forms which played a major role in coagulation. They were observed to transform into cells which occasionally were flattened and expanded. The cytoplasm became hyaline at the periphery, accompanied by an extreme flattening of the cell. The cell membrane spread out quite thinly as the cytoplasm expanded onto the glass surface. Granules became very apparent. The edges of the expanded, hyaline cytoplasm ranged from scalloped to slightly pointed extrusions or to extremely long, filiform hyaline extensions, which were frequently very active (Plate I, Figs. 12 and 13). These morphological variations occurred only after the preparation was several minutes old.

Rapid protoplasmic streaming was observed, with granules being carried along in the internal currents. Many hemocytes engaged in extension, retraction, probing, and anastomosis with similar strands. This process may have originated from the form with several cytoplasmic extensions or the fusiform-shaped cell as well. Further description of this process is given under "Coagulation," in the section following this one.

Adipohemocytes. A very distinct hemocyte was often observed, replete with lipoid droplets to the extent that the cell membrane was distended and the cell had a bubbly appearance (Plate I, Figs. 10B and 11). These were classed as adipohemocytes as defined by Jones (1962). They measured 10.9 μ ±1.1 μ, R = 5.6–17.4 μ,

FIGURE 3. Plasmatocytes of various shapes. Phase contrast. 700 ×.

FIGURE 4. Plasmatocytes with cytoplasmic extensions. Phase contrast. 875 ×.

FIGURE 5. Adipoid plasmatocyte with pseudopod-like extensions. Phase contrast. 875 ×.

FIGURE 6. Adipoid plasmatocyte with two lipoid inclusions. Phase contrast. 875 ×.

FIGURE 7. Small plasmatocyte with *Sarcina lutea* packets. Phase contrast. 1050 ×.

FIGURE 8. Disc-shaped and teardrop-shaped plasmatocytes. 3-D condenser-phase-objectives. 875 ×.

FIGURE 9. Spherule cells. A. Normal. B. Dead. Hyaline form with eccentric nucleus and "dancing" cytoplasmic particles. 3-D phase contrast. 875 ×.

FIGURE 10. A. Spherule cell in abnormal, elongated shape. B. Adipohemocyte. 3-D phase contrast. 875 ×.

FIGURE 11. Adipohemocyte. Phase contrast. 875 ×.

FIGURE 12. Flattening and expansion of a plasmatocyte on coverslip, showing granules in the cytoplasm. Phase contrast. 583 ×.

FIGURE 13. Plasmatocyte flattening onto coverslip and extending protoplasmic arms; cell membrane has become very thin and barely visible. Phase contrast. 583 ×.

FIGURE 14. Protoplasmic arm with well developed central body. Active streaming of protoplasm and movement of the body occurred. Phase contrast. 700 ×.

FIGURE 15. Same as Figure 14, taken about 10 seconds later. Bending of arms with central body as a fulcrum caused clump of cells to move in the preparation.

N = 22. Rhodamine B and Sudan IV were used to confirm the lipoid nature of these droplets. The frequency of these cells was always low, but increased during the prepupal stage and was correlated with the presence of free lipoids in the hemolymph. Complete transitional stages between an adipoid variant of a plasmatocyte with lipoid droplets and the hemocyte replete with lipoid droplets (adipohemocytes) were not observed.

Spherule cells. These were very large and highly refractile cells, round, oval, or distended by lobes of gray, homogeneous inclusions which appeared as dark clouds by phase contrast. Cells contained one to several of these inclusions, which distended the cell into irregular shapes (Plate I, Figs. 9A and 10A). Cells usually were unstable after about one hour *in vitro* and tended to rupture, ejecting amorphous material which rapidly dissipated. Some spherule cells never ruptured *in vitro*. After rupture, the nucleus, often previously obscured by the large inclusion, was very prominent, granular, and remained in the cell (Plate I, Fig. 9B). After intracellular disintegration the cell membrane was usually lined with granular debris. Measurements of 55 of these cells showed the longest axis to be 19.5 μ ± 9.8 μ (R = 11.9–34.7 μ) and the narrow axis measured 16.4 μ ±4.8 μ (R = 11.9–29.6 μ). Twenty nuclei measured 6.0 ± 1.0 μ (R = 4.1–8.1 μ).

The nature of the spherules is unknown. They did not fluoresce with any of the fluorescent dyes. Of the other stains used, only fast green FCF and Wright's stain were accepted. The spherules stained red with the latter stain.

In vitro changes of spherule cells occurred in direct preparations. Cells which had one or two medium-sized homogeneous gray inclusions transformed into large "mature" spherule cells. The cells lost their granular appearance, and the diameter increased as the typical gray refractile lobes appeared. The transformation was rapid or required up to 30 minutes in some preparations. Cells which looked like plasmatocytes were observed to develop inclusions which resembled spherules *in vitro*. However, plasmatocytes were not observed to develop into typical spherule cells.

Oenocytoids. No cells resembling descriptions for oenocytoids (Arnold, 1952; Jones, 1962; Yeager, 1945) were observed in wet-mount preparations, and all hemocytes could be placed into the above categories. Fixed, stained smears of boll weevil hemolymph did produce distorted cells which could have been mistakn for oenocytoids because of their uniformly staining cytoplasm, very small (shrunken) nucleus, and apparent creases or striations in the cell membrane. However, prior observation of the slides in wet-mount preparations revealed that these were artifacts. A few such cells were also seen in wet mounts diluted with phosphate buffer, pH 6.4, or in partially dried wet mounts not ringed with white petroleum jelly. Many cells in these mounts were distorted and could not be recognized. Cells resembling oenocytoids were never observed in preparations free from observable artifacts.

FUNCTIONAL PROCESSES

Phagocytosis. The plasmatocytes actively ingested foreign bodies. Larvae were injected into the hemocoel at the ventral side of abdomen with India ink, living *Escherichia coli* (Migula 1895) Castellani and Chalmers 1919, living *Sarcina lutea* (Schroeter 1886), or *S. lutea* stained with carmine. Hemolymph was drawn at intervals up to one hour after injection. All forms of plasmatocytes were seen with ingested particles. Living *S. lutea* was used most frequently because the packets were easily discerned in hemocytes (Plate I, Fig. 7). Hemocytes with spherule bodies were also observed with phagocytosed bacteria. The typical mature spherule cells never exhibited phagocytosis.

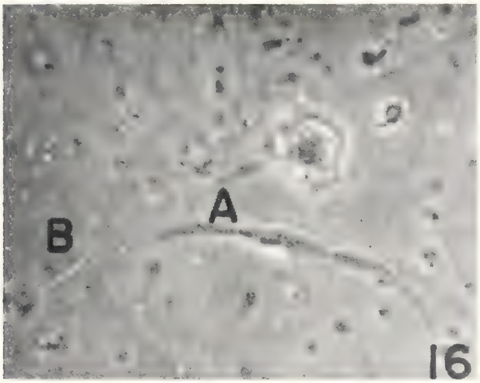
Coagulation. A phenomenon occurred *in vitro* which resulted in formation of extensive networks of protoplasmic strands interconnecting with hemocytes. The process, seen *in vitro,* was first observed 15 minutes to an hour after a preparation was made and proceeded slowly for several hours. Usually about an hour after activity was first seen the networks became quite extensive. One 17-hour-old preparation was a dense mass of closely interwoven plasma fibers and fibroblast-like structures connecting hemocytes, many of which still retained their normal shape. The process was first detected by the occurrence of filaments which often had a filiform extension. (The term "filaments" was used by Jones, 1962. See also Yeager, 1945, "plastids.") They were homogeneous except for one or two small black granules in the bulb-shaped body. These appeared suddenly in great numbers in preparations which later became very active in production of networks. These bulb-shaped filaments originated from plasmatocytes.

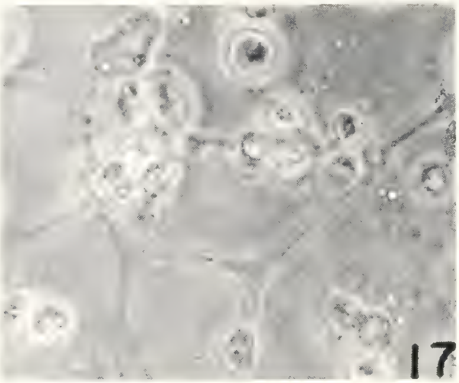
Disc-shaped plasmatocytes underwent transformations, observed most frequently in capillary-obtained hemolymph diluted about 1:1 with pH 6.4 phosphate buffer, in which they flattened and expanded, or proceeded to produce filaments or cytoplasmic extensions.

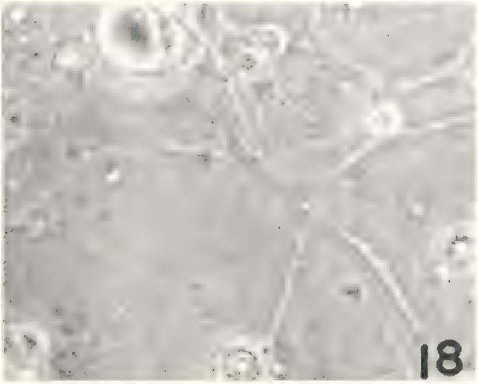
The most frequent event was production of one to four protoplasmic strands. After a long strand had been formed with a bulb-shaped thickening, further extension distally occurred. The entire strand exhibited active protoplasmic streaming and movement of the granules. The distal portion engaged in a rapid extension-retraction movement, along with a probing motion in all dimensions. Whiplike movement of the strands was common.

Two more events occurred simultaneously and represented the major phase of network formation (Plate I, Figs. 14 and 15). The proximal portion of the strand became thickened as cellular contents flowed into it. The thickened proximal strand changed frequently from a plastic to a turgid state and back again. However, the distal portion remained hyaline and continued to move rapidly. The amorphous bulb-like region was retained and a second such region often was formed in the proximal area of the strand. The flow of cellular content resulted in formation of a fusiform body in the strand, with granular inclusions and an additional body which by phase contrast resembled a nucleus.

The second concurrent event was anastomosis of the distal portions to form the network. The extremely active tips often extended 10–50 μ in one direction to form a terminal bulb-like body, which increased greatly in size but remained hyaline, and also sent out a second pseudopodial extension from the thickened body. Then both strands could be rapidly withdrawn while a third originated from a new location on the thickened body (Plate II, Fig. 16). When the two strands touched each other they recurved along their entire length and joined into a single larger strand. The pseudopodia could become quite rigid and were occasionally observed to force their way between cells clumped together and to separate a cell from the clump. Plate I, Figures 14 and 15 show such activity. The photographs were taken about 10 seconds apart. The result of the entire process was an extensive network in all dimensions, with newly formed thickened areas hardly distinguishable from fusiform plasmatocytes, and many areas of expanded protoplasm containing debris from parent cells. Extensive networks, formed one to two hours after removal of hemolymph and preparation of the slide, are shown in Plate II, Figures 17–19. More extensive formation frequently occurred after 6 to 17 hours.







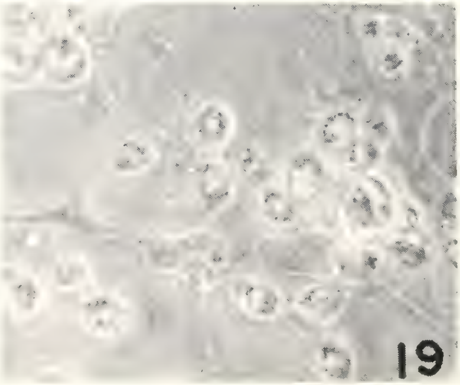


Plate II

FIGURE 16. Rapid recurving of two strands which have coalesced at A, and subsequent extension of a new strand at B. Phase contrast. 700 ×.

FIGURES 17, 18, 19. Extensive network formations 60-120 minutes after hemolymph withdrawal. Phase contrast. 350 ×.

Rupture of spherule cells occurred during this process and frequently was seen prior to the early stages of stranding and network formation. A definite relationship to initiation of the process by the erupted spherule cells was not established. However, protoplasmic stranding occurred in immediate proximity of the cells and even appeared to be attached to intact large spherule cells on several preparations. Their size and high refractivity prevented observance of the tenuous strands at the cell membrane, and no proof of actual connection was seen. From our observations, spherule cells do not initiate coagulation, nor do they play a role in coagulation process, with the possible exception of serving as a physical body to which the strands may attach.

One important factor directly influencing the extent of network formation was the proximity of the plasmatocytes to each other. Strands were frequently seen which had extended apparently to the maximum extent possible. If no other strands were encountered, further activity ceased. Anastomosis with other strands appeared to rejuvenate or increase the vigor of the strands and promote continued formation.

Effect of Physical and Chemical Treatment on Hemocytes

Hemocyte morphology and *in vitro* transformations were altered by the physical action of hemolymph withdrawal and placement on the slide and by dilution with pH 6.4 phosphate buffer, 0.85% NaCl solution, 0.5% (w/v) trehalose in distilled water (final pH 5.8), 1% potassium oxalate (w/v) in distilled water (final pH 6.75), or tap water (pH 7.3). Although quantitative data regarding the extent of the effect on hemocytes were not obtained, comparisons were made between hemocytes observed in direct preparations and those in capillary preparations or hemolymph diluted about 1:1 with the above diluents. Study of hemocyte morphology and *in vitro* transformations by several techniques of sample preparation provided an indication of pleomorphic variability and recognition of artifacts imposed by technique.

The physical action of drawing hemolymph into a capillary tube and then dispensing it onto a coverslip involves tremendous rates of flow at the orifice. The direct method for allowing an exuding drop of hemolymph to flow onto a coverslip is much less rigorous. This latter, direct, method was considered to result in hemocytes most like those in natural *in vivo* conditions. However, interpretation of observations from this method must also be tempered when one is attempting to draw conclusions as to the *in vivo* hemocyte conditions. Plasmatocytes and spherule cells were affected by the physical forces involved in physical withdrawal of the hemolymph. Changes in appearance of adipohemocytes and prohemocytes due to technique were never detected.

The capillary method resulted in great changes in plasmatocyte morphology. Very few plasmatocytes observed immediately after sample preparation by this method exhibited cytoplasmic extensions or were spindle-shaped. Disc-shaped forms were dominant and the flattened and expanded forms were frequent. On the other hand, preparation by the direct method resulted in immediate observation of all pleomorphic forms of plasmatocytes.

Dilution of hemolymph caused variations in hemocyte morphology. Although quantitative tests were not conducted, comparison of hemocytes in preparations diluted with the above materials with those in direct, undiluted preparations gave clear indication of artifacts. In the trehalose or potassium oxalate solutions, disc-shaped plasmatocytes were inflated and internal structural details more distinct. When the pH 6.4 phosphate buffer was used, disc-shaped plasmatocytes underwent a very rapid transformation to spindle-shaped and pseudopodial forms. However, dilution with water drawn directly from the tap (pH 7.3) did not result in this increased activity. Surprisingly, water from the central distillation source of the

Laboratory was found to be pH 6.1. Attention to the pH of diluents may be quite important in a study of hemocyte morphology.

DISCUSSION

The cells referred to as prohemocytes were not observed *in vitro* to change into mature cells. However, all intermediate stages from the typical prohemocyte described above to a spherical plasmatocyte were easily observed. As with all hemocytes, proof of origin may have to await application of tissue culture methods. Embryological study was not conducted. The reviews of Wigglesworth (1959) and Jones (1962) covered the origin of insect hemocytes. Imaginal discs, mitosis of hemocytes, and hemopoietic tissues have been reported as sources of post-embryonic replacement of hemocytes.

Plasmatocytes were the most abundant forms in all preparations. They were capable of extreme pleomorphism and varied considerably with regard to cytoplasmic inclusions. Close attention to nuclear size and placement, observation of *in vitro* changes, and intentional production of artifacts by various methods left no doubt as to the identity of all the observed forms. These hemocytes can carry on phagocytic activity, produce networks in a process which could be interpreted as coagulation, and possess different quantities and types of cytoplasmic inclusions. Because of transformation into many pleomorphic forms and variations of inclusion material, plasmatocytes are tentatively classed as a single category until further study proves other types to be valid end products of plasmatocyte differentiation.

The spherule cells are designated as a type of hemocyte because of their distinctive character. The slight phagocytic capability of spherule cells does not imply they are plasmatocytes because phagocytosis by pericardial cells and certain cells of adipose tissue has been reported (Cameron, 1934). Moreover, the function of phagocytosis has not been shown to be restricted to only one type of cell. Spherule cells were not demonstrated to play an active role in the coagulation process.

Adipohemocytes are considered a distinct type of cell because of their unique appearance and apparently unique function connected with lipid material. They were stable *in vitro* by all techniques employed. Although complete transitional stages from any other type of hemocyte, especially the adipoid plasmatocyte, were not observed, this does not establish the independent lineage of adipohemocytes. Further research may clarify the relationship between these hemocytes.

The process of coagulation was reviewed by Jones (1962) and Wigglesworth (1959). Grégoire (1951, 1955, 1959a, 1959b) studied the process in more than 400 species of insects. Four categories were described (Grégoire, 1951) and reviewed by Wigglesworth (1959). Type IV (no coagulation) was reported from two species of curculios (Grégoire, 1955). In a further study (1959b) he recorded Type IV from six additional species and observed the presence of hemocytes which exhibited the elongation, expansion, and retraction of flexuous protoplasmic arms, as well as formation of loose mesh-works of hemocyte protoplasm. In other studies (1959a) he reported network formation in many additional species of curculios. He also described "plastic hemocytes" which produced activity similar to that seen among plasmatocytes in *A. grandis*. Grégoire also reported prohemocytes, which probably correspond to those in the boll weevil, as well as other cells similar to boll weevil plasmatocytes. Filaments with thickened regions, were also reported to take part in network formation. In the boll weevil, similar filamentous bodies originated from plasmatocytes and were the terminal portions of protoplasmic strands which frequently dissociated from the cell during the process of network formation.

The process of network formation did not occur in every preparation. It was most frequent when the direct method was utilized and the preparation appeared to be normal, or lacking in artifacts. The proximity of cells undergoing stranding was important to the extent of network formation finally attained. When strands failed to contact others, the process was not as extensive as when cells were close enough to allow extensive contact and fusion, which appeared to rejuvenate the strands involved. The importance of the number of cells to coagulation was also observed by Yeager and Knight (1933).

A very graphic example of the importance of recognition of artifacts and study by several techniques occurred when a hemolymph sample was diluted with pH 6.4 phosphate buffer. We observed thin plasma strands with adherent particles and granular plasma islands identical to those pictured by Grégoire (1951, Plate V, Figure 25). We saw this condition only once, and our attempts to repeat it were unsuccessful. Although Grégoire did not dilute the hemolymph, he allowed it to flow by capillary action between an unsupported coverslip and the slide. This incident serves merely to illustrate the necessity for studying hemocytes by several methods and attempting to recognize the possible alterations caused by technique and physical or chemical conditions.

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SUMMARY

1. Hemocytes of larval boll weevils, *Anthonomus grandis* Boheman, were classified on a morphological basis into four types. *Prohemocytes* had a large nucleus and a thin band of peripheral cytoplasm, which increased in quantity and became more optically dense to phase contrast as the prohemocytes matured. All gradations to spherical *plasmatocytes* were observed. *Plasmatocytes* were characterized by their great pleomorphic capability. The cytoplasm varied in that it was granular with fine or large granules, dense and uniform to phase contrast or heterogeneous with areas of different optical density as well as possessing vacuoles or various inclusions, in addition to being hyaline or refractive at the cellular periphery. Plasmatocytes assumed spherical, pseudopodial or fusiform, as well as irregular, shapes during a process of stranding or when flattening onto a glass surface. Plasmatocytes were phagocytic. Cells filled with lipoid globules were tentatively classed as *adipohemocytes.* A fourth type of cell, *spherule cells,* was characterized by the presence of one to several large globules which were amorphous and non-refractive to phase contrast. These cells were large and often distended by the inclusions.

2. A slow process of network formation was the only observed indication of coagulation. Plasmatocytes underwent extension stranding by extension, retraction,

probing, and anastomosis with protoplasmic arms from other cells to produce networks. The extent of these networks depended partially upon the proximity of stranding cells.

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AET AS A RADIOPROTECTIVE AGENT AT THE CELLULAR LEVEL[1]

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Many agents have been tested for their possible protective effect against the damaging and lethal action of ionizing radiations (Rugh, 1953a, 1958; Hollaender and Doudney, 1954; Thomson, 1962). Among the most effective agents for the mature mammal are AET (S,2-aminoethylisothiourea-Di-HBr), MEG (2-mercaptoethylguanadine), MEA (B-mercaptoethylamine) and cysteine HCl (Doherty and Burnett, 1955, 1961; Stern, 1956; Doherty *et al.*, 1957; Doherty and Shapira, 1958; Maisin and Doherty, 1960; Maisin and Popp, 1960; Maisin, 1961; Mundy *et al.*, 1961; Dacquisto *et al.*, 1961; Blouin and Overman, 1962; Melville and Leffingwell, 1962; Ehling and Doherty, 1962; Hanna and Colclough, 1963; Mittler, 1963). AET has also been shown to be protective against the effects of the so-called radiomimetic agents (Asano *et al.*, 1962, 1963). In every case the drug used, to be effective, had to be administered prior to exposure to the ionizing radiations. Usually such agents, injected after irradiation, were deleterious. Only spleen and bone marrow homogenates appear to have any protective value when administered after exposure (Bacq and Alexander, 1961).

The usual explanation for the so-called protective action of any of these agents is that they somehow aid in hematopoietic recovery (Dickens and Shapiro, 1961; Colclough and Hanna, 1963) or cause hypoxia (Hanna and Colclough, 1963). How protection is actually accomplished is not at all clear.

In order to understand better the mechanism of protection, it seemed wise to test the most effective agent, AET, at the cellular level. For this study the unfertilized egg of the sea urchin, *Arabacia punctulata,* was used. Thus, any irradiation and possible radioprotective action of AET on the haploid cells would be reflected in the early cleavage stages, as well as in early organogenesis. This is a report of the findings.

MATERIALS AND METHOD

The eggs of *Arbacia punctulata* were obtained from 5 or 6 mature females by cutting away the Aristotle's lantern from the oral region and inverting them in stender dishes of filtered sea water, and allowing them to shed naturally through the five gonopores. Such eggs were washed in two changes of filtered sea water, 15 minutes apart, and allowed to settle. When the eggs were to be subjected to AET, two times the desired concentration of the agent was used, added to an equal volume of egg suspension, resulting in the proper concentration of AET.

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All eggs were fertilized simultaneously by a sperm suspension made from a single sea urchin.

The radiation facilities consisted of the cesium-137 paired sources available at the Marine Biological Laboratory, Woods Hole, Mass. The eggs were suspended midway between the two sources of these gamma rays at a distance of 5.9 cm. from each source and a dose rate of 5000 r/min. After some exploratory tests a total exposure of 50,000 r was chosen, delivered in 10 minutes.

The AET was obtained from Dr. D. G. Doherty of Oak Ridge National Laboratory, and was identical with that used by him so successfully with mammals (Doherty *et al.*, 1957), and was kept in a desiccator. In order to convert the AET into the effective MEG, it was first dissolved in 10 cc. of phosphate buffer at pH 7.4, before diluting with 490 cc. of filtered sea water, resulting in pH range from 7.3 to 7.6. This is known to be optimum for the normal development of *Arbacia* (Harvey, 1957). The concentration of AET to be used was empirically determined, being the threshold level just below toxicity (see below). The solution thus provided was largely MEG which had to be used within 30+ minutes, before its oxidation degradation to the disulfide.

All studies were made at the laboratory temperature, which ranged from 21.5° to 23.0° C., but was uniform for any single set of experimental variables. *Arbacia* samples were fixed in 10% formalin in filtered sea water at 20-minute intervals, beginning one hour after fertilization when the controls normally show a high percentage of cleavage. Percentages were based upon a minimum of 200 cell counts, the data presented here (Table I) being on 400 cell counts.

When eggs were subjected to AET this was done for a minimum of 10 minutes prior to irradiation in order to bring this agent into equilibrium with the egg substance. When *Arbacia* eggs were returned to sea water from the AET solution they were diluted with approximately 100 times the volume of filtered sea water.

Experimental Data

Exploratory experiments indicated that exposures of the unfertilized eggs of *Arbacia* to 5000 r or more resulted in cleavage delay and 50,000 r still allowed 11% to cleave, after considerable delay over the controls. Some 18% showed anomalies during the early cleavages. None achieved the pluteus stage, nor even gastrulation. Thus, if AET were able to "protect" such irradiated eggs this would be revealed in a shortening of the time from fertilization to the first cleavage, an increase in the percentage of cleavage within a given period, reduction in the incidence of anomalies and/or effects on development past gastrulation. This exposure of 50,000 r did not render the eggs unfertilizable by normal sperm and they readily formed fertilization membranes. There was some evidence that these membranes were not fully elevated, and that the perivitelline space was not as wide as in the controls, and the eggs were often clustered as if they were sticky (Rugh, 1953b). Lower exposures did allow a higher percentage of cleavage, and further development, but the 50,000 r level of exposure was chosen since the low level of 13% cleavage could be improved if there were any protection. In 13 separate sets of data, involving thousands of eggs, only 5 or 6 exposed to 50,000 r were able to achieve the pluteus stage, and not many more survived the process of gastrulation. This made the end point of development, following 50,000 r gamma rays, clear-cut.

The AET was neutralized to pH 7.3–7.6 but was found to be toxic for *Arbacia* eggs in concentrations comparable to those used for mammals. Membranes were elevated, asters were formed, and cleavages did occur in concentrations up to 60 mgm.% (2% cleavages). However, since as little as 5 mgm.% AET in sea water destroyed the developing *Arbacia* eggs some time after fertilization and early cleavage, if left in the solution, it was decided to use 3 mgm.%, which allowed 97% of the unirradiated eggs to be fertilized and cleave normally, both with respect to time and form. A lower concentration of 1 mgm.% allowed *Arbacia* eggs to be fertilized and to develop, similarly with the controls, through the pluteus stage. There appeared to be no evidence that this concentration was in any way toxic. The concentration of 3 mgm.% was therefore chosen for use prior to and during irradiation since, in a few experiments, this concentration did appear to have a very slight delaying effect on the time of the first cleavage if the eggs were left in the AET solution. This concentration was therefore considered to be at the threshold level of toxicity. The crucial experiment, based upon these empirical findings with irradiation and AET concentrations, was exposure of the eggs for 10 minutes prior to and 10 minutes during gamma irradiation (50,000 r) to the 3 mgm.% solution of AET, and then transferring them to filtered sea water for fertilization and development. Continuing exposure of the irradiated eggs to AET allowed slight improvement in the time and percentage of cleavage after fertilization; only 20% became ciliated blastulae and all were dead by 48 hours. Thus, it seemed that the optimum conditions involved return of the irradiated eggs from the AET to filtered sea water for fertilization and development.

The data of the final experiment alone will be given since they followed the general pattern of the prior experiments and had higher counts for each of the variables, of 400 cells per sample.

The data of the table above substantiate Henshaw (1932, 1940) and Henshaw and Francis (1936), who report that a delay in fertilization of irradiated and unfertilized eggs of *Arbacia* allows some of them to "recover" so that the retardation in the initial cleavage normally caused by irradiation is somewhat nullified. The percentage difference is not great, after 50,000 r exposure, but the eggs irradiated at the beginning of the series started to cleave at a shorter elapsed time interval than those irradiated at the end of the series, due to the recovery phenomenon of Henshaw. Eggs in 12 mgm.% AET showed reduced percentage of cleavage throughout, and by 24 hours virtually all were dead. Likewise, those irradiated in 12 mgm.% but returned immediately to sea water for fertilization and development showed no "protection" by this agent, and all were dead by 24 hours. In 3 mgm.% AET the results were comparable to the controls, indicating no significant delay or deleterious effect on cleavage or development. When eggs were irradiated in 3 mgm.% AET and immediately returned to sea water for fertilization, there was no improvement in cleavage time in relation to those irradiated without the drug. By 24 hours only 30% became ciliated, and were retarded in development. As expected, 1 mgm.% AET had no effect on either fertilization or development. Thus, while AET in all concentrations used allowed fertilization of the treated eggs, and 3 mgm.% was definitely non-toxic, no concentration used prior to and during irradiation had any "protective" effect, either on the time of the initial cleavage, gastrulation or later development. While 3 mgm.% AET allowed unirradiated eggs to be fertilized and develop (by 48 hours) to motile plutei, no eggs irradiated in AET to 50,000 r gamma rays ever reached the pluteus stage, or even gastrulation.

TABLE I

Cleavage % (400 eggs) at intervals after fertilization

Conditions	60′	80′	100′	120′	24 hrs.	48 hrs.
Controls	74%	92%	94%	99%	Cil. gastrula	Norm. plutei
50,000 r gamma rays	0	0	2	11	30% cil. blastula	0.1% cil. blastula, no plutei
12 mgm. % AET only	25	32	36	49	30% alive, retarded	20% alive, abnormal, retarded
12 mgm. % AET + 50,000 r + sea wate	0	0	1	4	99% dead	0.1% cil. blastula
3 mgm. % AET only	72	90	95	99	Cil. gastrula	Norm. plutei
3 mgm. % AET + 50,000 r + sea water	0	0	1	7	30% cil. blastula	0.1% cil. blastula, no plutei
3 mgm. % AET + 50,000 r + AET	0	0	9	21	30% cil. blastula	100% dead
1 mgm. % AET only	69	90	96	99	99% cil. gastrula	Norm. plutei
50,000 r gamma rays*	0	0	0	2	10% cil. blastula abnormal	100% dead

*Unfertilized eggs were irradiated at the beginning of the series and another group at the end (30 minutes later) but all were fertilized simultaneously so that the first group had 30 minutes "recovery" time, due to delay in fertilization. The effect of the delay is reflected in the slight difference in cleavage data.

DISCUSSION

"Protection" in radiobiology is really a misnomer. Its limitations should always be clearly defined by the user. Even among mammals there are only a few agents which, if present in the body at the time of exposure, will provide greater tolerance of whole body exposure to ionizing radiations, resulting in a higher LD/50/30 (*i.e.*, lethal dose to 50% of exposed animals in 30 days). But this is *survival*, and, to that extent, protection against death. The tendency is to assume that all animals that survive a 30-day post-irradiation period have "recovered" from any and all ill-effects of the exposure and are as normal as they were prior to the exposure. This is simply not true. Whole body exposure takes a toll which can be expressed in a variety of sequelae, and some effects are irrevocable, irreparable. This is not to belittle the possible clinical importance of devising means to extend survival to a larger population of exposed individuals, no matter how they fare in their survival. But we must not be blinded to the sequelae of such exposure and survival.

The mechanism of "protection" by AET solutions has been elusive to many investigators. It has been thought to function through aiding in hematopoietic recovery, but the same agents which seem to do this are deleterious if given after irradiation. It has been suggested that AET might help to remove toxic radicals before they can injure normal cells, or in some way to actually stimulate cell proliferation in specific tissues. While all theories have been vague, it has been presumed that any "protection" should ultimately be demonstrable at the cellular level. Tied in with protection of the adult mammal (Khym *et al.,* 1957) is the demonstrated fact that genes and chromosomes are particularly radiosensitive. Recently, Mittler (1963) found that neither AET nor MEA had any protective effect against induced mutations or translocations in chromosomes of *Drosophila* spermatocytes or spermatids. But AET has been the most effective agent with mammals so that this present study seemed imperative.

There is a wide species variation in the toxicity levels and the responses to AET (Colclough and Hanna, 1963), ranging from 10–20 mg./kg. given orally to man, to 640 mg./kg. injected into mice (Doherty, personal communication). Its chemistry and degradation products are well documented by Doherty and his co-workers. Since it is not a highly stable compound, except under rather rigid laboratory conditions, it may not prove to be as universally useful as might be desired. Nevertheless, among the hundreds of chemical agents tried it now appears to be the most effective for most mammals.

The *Arbacia* egg is so well known, so dependable in its reactions to almost all environmental variables, that it proves to be an ideal haploid cell on which to test the efficacy of any possible radioprotective agent. The several criteria of effect are: fertilizability, membrane elevation, aster formation, cleavage time, cleavage form, blastula-gastrula transition, and plutei formation. Each successive criterion is the more sensitive, as development proceeds, and if the pluteus stage is attained one may consider survival to be quite complete, at least as complete as 30-day survival for the mammal.

Ionizing radiations (x-rays) have been shown to have a direct effect on the cleavage mechanism of *Arbacia* so that, with increasing exposures (within limits) there is increasing delay in the time of the first cleavage (Henshaw, 1940). Recently Rao (1963) demonstrated that this was due to chromosome condensation interfering with the mechanism of mitosis. Time appears to heal, somewhat, irradiation damage because a delay in the fertilization of irradiated eggs reduces the effect of that irradiation on the initiation of the first cleavage. This was originally referred to as "recovery" (Henshaw, 1940), but was more recently shown not to be full recovery but rather a return toward the normal cleavage times (Rugh and Wolff, 1956; Rugh, 1958), with the ultimate embryonic death unaltered. This recovery of cleavage time was nevertheless of interest in view of the finding (Rugh, 1950) that ionizing radiations so affect (meiotic) chromosomes that they become sticky and are permanently clumped. Delay in fertilization could hardly disentangle fused chromosomes, although such delay could allow time for the re-fusion of fragmented chromosomes. Henshaw (1938) long ago showed that the delay in cleavage time seen in irradiated eggs was not seen in the enucleated fragments of *Arbacia* eggs, so that this phenomenon is directly related to chromosome effects. Apparently it is the early prophase stage which is so affected, and such delay is not carried over into subsequent cleavages (Yamashita *et al.*, 1939).

In order to avoid confusing any possible effect of AET with the "recovery" in normal cleavage time due simply to a delay in fertilization of the egg, control-irradiated eggs were provided at the beginning and at the end of each experimental series. Thus, the maximum "recovery" of cleavage time would be demonstrated by the first control-irradiated eggs (which had maximum delay in fertilization) while the maximum damage from irradiation would be shown in the cleavage percentages of the final group, fertilized immediately after irradiation. Since the temperature was uniform for any set of variables of any single experiment, this factor could be ruled out.

AET was positively *not* protective to the egg of *Arbacia,* using any of the criteria listed above. This does not rule out the possibility that some other agent(s) might be "protective" in one or another sense. Certainly the mechanism of the so-called "protection" need not be identical for all cells, tissues, organs or organisms, but this agent, so useful with the mammal, is totally without benefit to the gamma-irradiated haploid egg cell of *Arbacia,* prior to fertilization.

SUMMARY AND CONCLUSIONS

1. AET (S,2-aminoethylisothiourea-Di-HBr) was used in a concentration just below the threshold toxicity level, to determine whether it might afford any radioprotection for the haploid *Arbacia* egg exposed to 50,000 r gamma radiation prior to fertilization with normal sperm.

2. *Arbacia* eggs could be fertilized in 3 mgm.% AET and would cleave and develop, both in time and manner comparable to the controls in pure sea water. Toxicity was indicated above 5 mgm.%, particularly after irradiation.

3. *Arbacia* eggs exposed to 50,000 r gamma rays showed a delay in the initiation of the first cleavage with ultimate cleavage reaching only 11% and abnormalities reaching 18%. Not a single egg so exposed ever reached the pluteus stage. The delay in the initiation of the first cleavage was also reduced by a delay in fertilization, and the percentage of ultimate cleavage was improved.

4. The optimum conditions provided were: Exposure to 3 mgm.% AET in sea water for 10 minutes prior to and 10 minutes during gamma irradiation to 50,000 r, and yet this allowed no improvement in cleavage time, degree of membrane elevation, or development. Not a single egg thus treated reached either the pluteus or gastrula stages.

5. It is concluded that while AET has proven to be radioprotective for the adult mammal, this protection (survival) may not be effected through individual cells but through tissue or organ regeneration. However, extrapolation is always hazardous and AET may be cell- or species-specific. The haploid *Arbacia* cell (cytoplasm and nucleus) is not subject to any protective action from AET.

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HEAT TOLERANCE AND TEMPERATURE RELATIONSHIPS OF THE FIDDLER CRAB, UCA PUGILATOR, WITH REFERENCE TO BODY COLORATION

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Fiddler crabs, *Uca,* being inhabitants of the littoral zone, offer a ready opportunity for study of physiological adaptations associated with the intermediate step in the migration of crustaceans from an aquatic to a terrestrial habitat. Poikilotherms, in general, that have left the aquatic environment require, among others, adequate mechanisms to survive greater extremes of temperature than they had experienced previously. Fiddler crabs are generally active and feeding about the time of low tide. Consequently, when low tide occurs in the middle of the day during the warmer months of the year fiddler crabs on open beaches are exposed to temperatures that are near the lethal level because of the direct solar radiation (Teal, 1958).

The fiddler crab, *Uca pugilator,* exhibits a daily rhythm of color change (Abramowitz, 1937). Specimens are darker during the daytime than at night because the chromatophoral pigments are more dispersed. Brown and Sandeen (1948), working with the same species, found that during the day phase of the rhythm the black pigment tends to concentrate as the temperature rises above 15° C. but the white pigment tends to disperse even further as the temperature increases above 20° C. These responses, which are superimposed on the daily rhythm, would appear to have a thermoregulatory function because during intense sunlight concentration of black pigment would diminish the area which maximally absorbs radiant energy, whereas dispersion of the white pigment would increase the area which reflects this radiation. However, on no crustacean have reflectance measurements been actually performed. Furthermore, no investigator has compared body temperature of pale and dark crustaceans.

Fiddler crabs have been used in a few temperature tolerance studies, but Edney (1961) is the only other investigator who has measured their body temperatures under experimental conditions. Orr (1955) reported the time required to kill all the crabs, *Uca pugilator,* at each of several different temperatures. Teal (1958) determined the upper temperature that was lethal to 50% of the specimens from each of three species of fiddler crabs, *Uca pugilator, U. minax,* and *U. pugnax.* The values were 39.5°, 39.9°, and 40.0° C., respectively, but the differences were not statistically significant. Vernberg and Tashian (1959) reported that a tropical zone species, *Uca rapax,* was more resistant to temperatures of 42° and 44° C. than was a temperate zone species, *Uca pugnax.* Edney (1961), who studied the water and heat relationships of five species of *Uca* from Mozambique, found that those species from which water transpired faster generally had the lower body temperatures.

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The general purpose of the present investigation was to provide further information about the thermal and water relationships of the fiddler crab, *Uca pugilator*. The specific aims were to determine (a) the effect humidity has on body temperature and survival at high temperatures, (b) the influence of body coloration on body temperature, and (c) the rates of transpiration at high temperatures.

MATERIALS AND METHODS

Adult male and female specimens of *Uca pugilator* were collected for use in these experiments at Grand Isle, Louisiana, and at three locations in the area of Woods Hole, Massachusetts; namely, Chapoquoit Beach, Great Sippewissett Creek, and Great Lake. Crabs from Grand Isle were used only for the reflectance study described below. The crabs were maintained in the laboratory in wooden tubs and stainless steel tanks containing sea water. Each container was inclined so that the bottom was not completely covered with water. The sea water was changed every second or third day. The crabs were fed fish which had been cut into small pieces.

To determine the heat death point in saturated air, 10 crabs were placed into each of six 500-ml. Erlenmeyer flasks containing 20 ml. of sea water. Aside from their legs a very small percentage of the body of each crab was in contact with the water. Crabs were chosen without regard to sex because in a preliminary series of experiments no difference between the resistance of the sexes was apparent. The temperature of the water was at the desired value before the crabs were put in. The flasks containing the crabs were quickly immersed completely for one hour in one of a series of constant temperature water baths maintained at the desired temperature. The air above the heated water was ascertained to be 98–100% saturated by directing a stream of air from the flasks over a wet-dry bulb hygrometer suspended in a plastic tube 4 cm. in diameter. After the hour the crabs were removed from the flasks, placed into glass fingerbowls containing sea water, and allowed to stand at room temperature for 12 hours, at which time the number of survivors was ascertained. Any crab which was unable to right itself was not considered a survivor.

Determinations of thermal death limits in dry air were performed through use of an apparatus adapted from the one described by Edney (1951a). This apparatus also allowed determination of transpiration rates. Since surface area was important in the measurement of transpiration, only males were used in this particular set of experiments. Ten crabs were subjected simultaneously to a stream of slowly moving air at a constant predetermined temperature. The air which displaced one liter of water per minute from an inverted graduated cylinder was dried by passage through two chambers of Indicating Drierite. The procedure was performed at six different temperatures.

When body temperatures were determined, as during exposure to sunlight in the habitat, crabs were suspended just high enough over a white sand background so that their legs could not touch the substrate. Each crab was held in position by a tin clamp which gripped it at the lateral edges of the cephalothorax. The clamp was designed to shield a minimal area of the dorsal surface. Body temperatures were measured by means of a Brown Portable Potentiometer (Model 126W2) modified to supply eight leads and thermocouples of fine gauge copper and constantan wires. The thermocouples were forced into the crabs at the ventral junction of the cephalothorax and abdomen to a position near the midgut.

The specific reflectance of visible light was determined for pale and dark crabs through use of a General Electric Recording Spectrophotometer. The courtesy of the personnel at the Southern Utilization Research and Development Laboratory of the U. S. Department of Agriculture, New Orleans, Louisiana, in making this apparatus available is gratefully acknowledged.

To determine the effect of transpiration alone on the body temperature, crabs in an apparatus adapted from Edney (1951b) were exposed to a stream of slowly moving air of constant temperature which displaced one liter of water per minute. The relative humidity of the air could be adjusted to 0%, 50%, and 100% saturation by passing it through a single chamber or a combination of chambers each containing Drierite, water, or a sulfuric acid solution (Solomon, 1951). The apparatus, including the humidity control chambers, was immersed in a constant temperature bath for each determination. The crabs used in this experiment were clamped in such a position that they were unable to touch the sides of the apparatus.

The rate of transpiration at each of a series of constant temperatures was determined with male crabs each weighing more than two grams through use of the apparatus described above. All crabs were blotted and weighed at the start and weighed one hour later at the completion of the experiment. Data for crabs that defecated during the course of the experiment were discarded. The rate of water loss due to evaporation was expressed as milligrams of water lost per square centimeter of surface area per hour. Surface area was determined by flattening the exoskeletons of a number of weighed crabs, tracing the outlines, and determining their total areas with a planimeter. Then through use of the equation $S = KW^{2/3}$ (Wigglesworth, 1945) a constant could be determined for use with the experimental crabs. The "k" value of adult males was found to be 10.56.

Experiments and Results

Thermal tolerance in dry and saturated air

Groups of crabs were exposed in the manner described above to one of six constant temperatures ranging from 37° to 42° C. in saturated air, and to one of six constant temperatures ranging from 40° to 47° C. in dry air. The experiment with saturated air was performed with a total of 50 crabs at each temperature and in dry air with a total of 20 crabs at each temperature. The percentage survival at each temperature for both groups of crabs is plotted in Figure 1. These experiments were performed during the first two weeks of August, 1960. The temperatures lethal to 50% of the crabs in saturated and dry air are indicated by the dashed lines in Figure 1: 40.7° C. and 45.1° C., respectively. This appreciable increase of the lethal temperature would be expected if crabs in dry air are able to maintain a body temperature below that of crabs in saturated air where transpiration cannot occur. Most of the water loss must have been from the gill surfaces and gill chambers. The lethal temperature for crabs in the field would be expected to lie between the values determined in saturated and dry air and would certainly be approached on hot, clear days.

Effect of humidity on body temperatures

Crabs were exposed in the laboratory first to dry air, then air 50% saturated, and finally fully saturated air. The air temperature was 37.5° C. Air and body temperatures were recorded every five minutes. The humidity was not changed until the body temperature remained constant for 20 minutes. The results are shown in Figure 2. This experiment was performed 21 times. Inspection of the figure reveals a direct proportional relationship between the increase in body temperature at equilibrium and the increase in relative humidity.

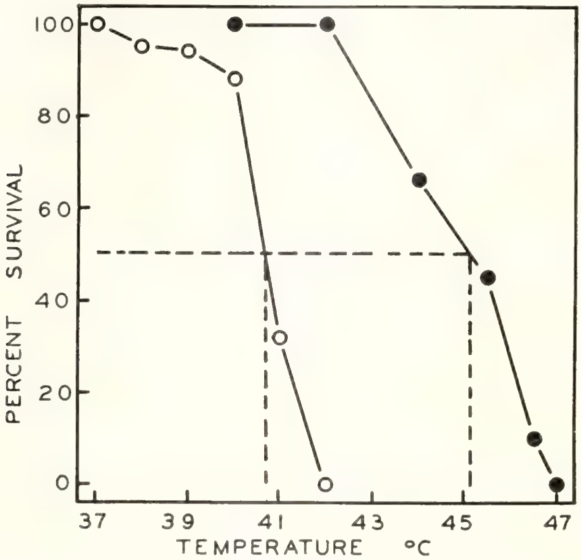

FIGURE 1. Relationships between the temperature to which fiddler crabs were exposed for one hour in saturated air (circles) and in dry air (dots) and the percentage surviving.

In Figure 3 are shown (a) the mean rates of transpiration of water from male crabs in dry air from 28° to 48° C., and (b) the saturation deficit of dry air at each of the experimental temperatures. At each temperature the rate of transpiration was determined for each of 20 crabs. The saturation deficit values were taken from the Handbook of Chemistry and Physics, Fourth Edition. The increase in rate of evaporation with increase in temperature is proportional to the saturation deficit of the air. The similarity of the curves shows that transpiration of water from these fiddler crabs is a physical process completely dependent on the saturation deficit. The crabs apparently have no control over the rate at any temperature.

Effect of coloration on body temperature

This experiment was devised to test the hypothesis that the blanching of fiddler crabs at high temperatures, first described by Brown and Sandeen (1948), has thermoregulatory significance. Dark crabs having maximally dispersed melanin and pale ones with maximally concentrated melanin were taken from the laboratory

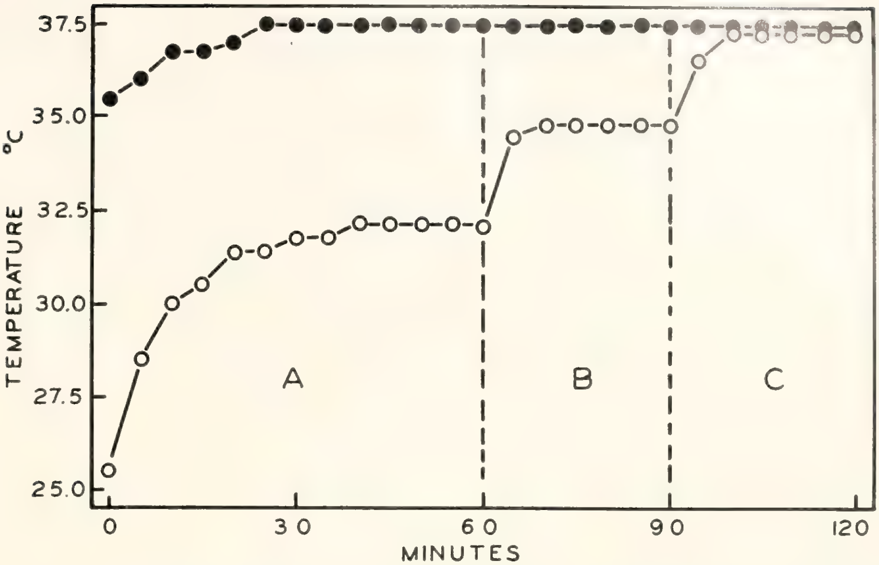

FIGURE 2. Relationship between mean body temperature of crabs and time of exposure to slowly moving air of (A) 0%, (B) 50%, and (C) 100% relative humidity (circles). Air temperature is indicated by the dots.

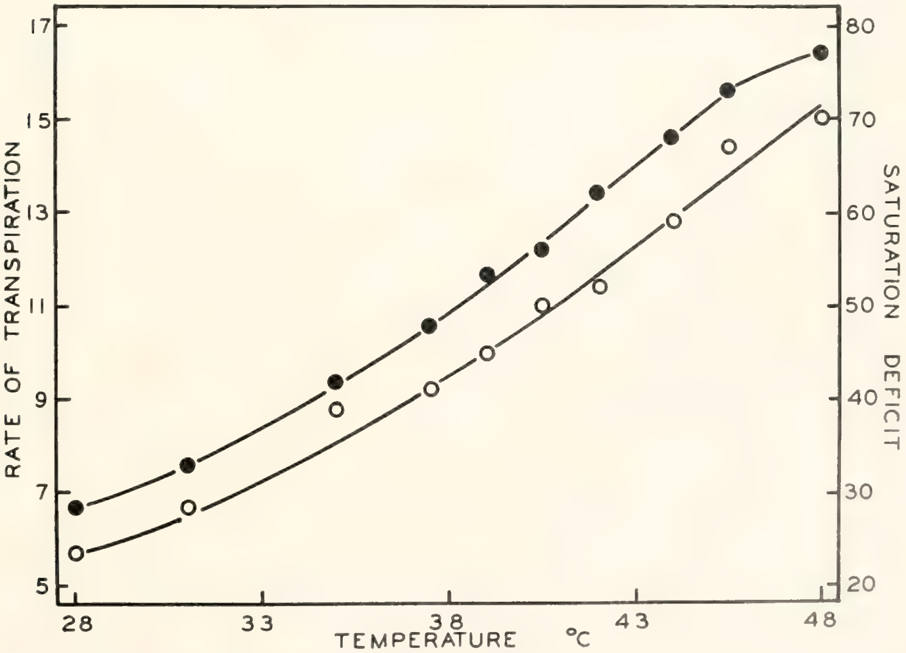

FIGURE 3. Relationships between (a) rate of transpiration from fiddler crabs (mg./cm.^{2}/hr.) and temperature (circles) and (b) saturation deficit (mm. Hg) of dry air and temperature (dots).

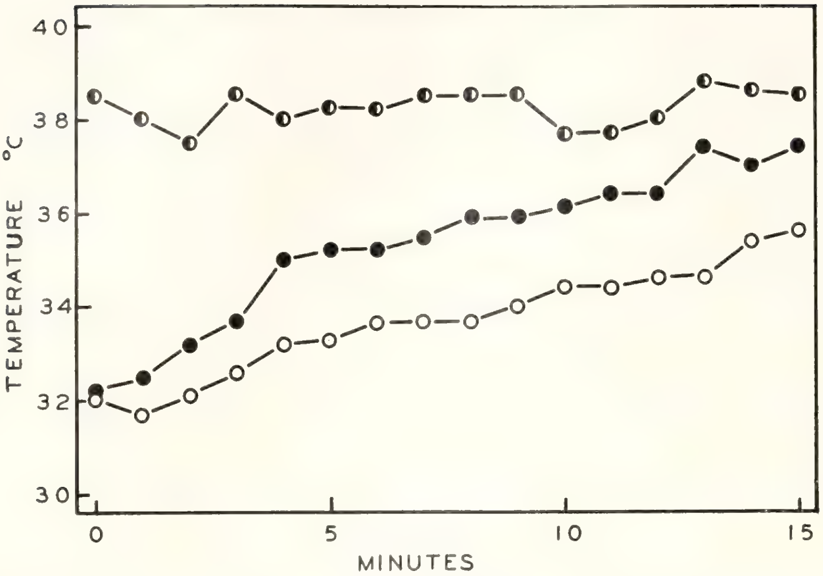

FIGURE 4. Relationships between body temperature of pale (circles) and dark (dots) fiddler crabs and time of exposure to sunlight. Substrate temperature is indicated by the half-filled circles.

and exposed to sunlight on a bright, almost windless day. Their body temperatures were recorded over an interval of 15 minutes while they were clamped in position. The data are presented in Figure 4 where each point represents the mean of 10 determinations. The effect of color differences in absorbing and reflecting radiant

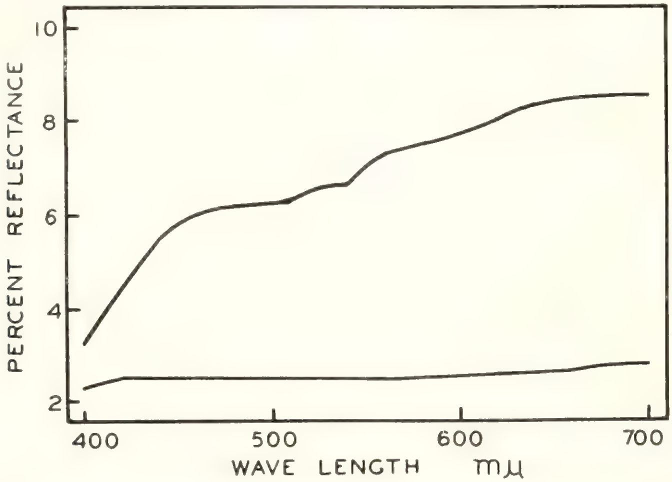

FIGURE 5. Relationships between percentage of light reflected from the dorsal surface of the cephalothorax of pale (upper curve) and dark (lower curve) crabs and the wave-length of light.

energy and converting it to heat is evident in the difference between the rates of heat gain of the pale and dark crabs. Within five minutes after the crabs had been set in sunlight the dark crabs had a body temperature 2° C. higher than that of the pale crabs.

At each of several wave-lengths of visible light from 400 to 700 mμ, the quantity of light reflected from the dorsal surface of the cephalothorax was greater with pale crabs than dark ones (Fig. 5). The difference at the longer wave-lengths was as much as 5%. Furthermore, for both the pale and dark crabs the percentage of light reflected was greater at the longer wave-lengths than at the shorter ones although the contrast between the shorter and longer wave-lengths was more readily apparent with pale crabs than with dark ones.

Observations of field behavior of specimens of Uca pugilator

Field observations of the activity and behavior of natural populations of *Uca pugilator* were made throughout July and August, 1960, at Chapoquoit Beach and Great Sippewissett Creek. Observations of individual crabs were made from a distance through binoculars. The days chosen were clear and hot with a minimum of wind (1 on the Beaufort scale: direction of wind shown by smoke drift only, Meteorological Observer's Handbook, 1942). The air temperature averaged 27.5° C. at a height of 2 cm. above the ground. The surface, dry sand, averaged 42.5° C. The air temperature in burrows in this dry sand 2 cm. below the surface averaged 30° C. and 15 cm. below the surface was 26° C. Excavation revealed that all burrows ended in very damp or wet sand. The average relative humidity on the days of observation was 60%, determined with a sling psychrometer held 60 cm. above the surface.

On hot days crabs were observed actively feeding predominantly in areas where the surface of the sand was still moist; however, occasionally they were also active on the drier parts of the beaches. In their normal course of activity the crabs would feed for a while and then retreat into their burrows. It was observed that each crab would feed for 15 to 20 minutes at a time, then disappear into a burrow for three or four minutes and reappear to resume feeding. Therefore, the frequency of this "feeding-retreat" rhythm is 18–24 minutes. The crabs did not enter their burrows *en masse* as they do when startled, but each individual adhered closely to its own cycle.

Discussion

The lethal temperatures indicated in Figure 1 at which 50% of the crabs died are more meaningful statistics than are the temperatures at which 100% of the crabs died. The latter temperatures must necessarily include even the most resistant individuals. Teal (1958) found that 50% of the fiddler crabs, *Uca pugilator,* that he used died after they had been exposed for one hour to a temperature of 39.5° C. "by placing the animals in enough sea water of the desired temperature so that each animal was slightly more than half covered." The lowest corresponding value found in the present set of experiments was 40.7° C. with crabs in saturated air. This difference is probably a reflection of the difference in experimental technique because in the present experiments 10 crabs and only 20 ml. of sea water were placed into a 500-ml flask, with the result that very little submergence of the crabs occurred. It will be recalled these flasks were immersed completely in a constant temperature water bath but Teal did not state how he kept the temperature of his flasks constant.

The data presented herein confirm and extend the observations of Edney (1961) on five species of African fiddler crabs. He found, as herein, that (a) the temperature within the burrows during the warmer months of the year is considerably cooler than the sand at the surface, and (b) an appreciable reduction in body temperature occurs as a result of transpiration. The ability to withstand high surface temperatures by transpiration undoubtedly has a significant survival value. The upper lethal temperatures at which all of Edney's crabs died ranged from 42° to 45° C. for the five species after immersion in water of the appropriate temperature for 15 minutes. With one hour of exposure in saturated air the temperature required to kill 100% of the *Uca pugilator* herein was 42° C. which is the same temperature that was lethal to all of Edney's specimens of *Uca marionis, U. urvillei,* and *U. chlorophthalmus.* Edney obtained these particular data in January, 1959. Inhaca Island, where he worked, is south of the Equator, rendering January one of the warmer months of the year for these crabs. It was shown herein for the first time with a fiddler crab that (a) the amount of cooling resulting from transpiration is proportional to the decrease in relative humidity (Fig. 2), and (b) the "saturation deficit law" as stated by Edney (1957) is obeyed (Fig. 3).

The results shown in Figure 4 support the hypothesis of Brown and Sandeen (1948) that the blanching of fiddler crabs at high temperatures has a thermoregulatory role. Pale crabs maintained themselves about 2° C. cooler than dark crabs. The measurements of quantities of visible light reflected from pale and dark crabs (Fig. 5) are qualitatively similar to the findings of Deanin and Steggerda (1948) with pale and dark frogs. However, Deanin and Steggerda did not measure the body temperatures of their frogs but they did point out that the greater reflection of light at the red end of the visible spectrum than at the violet end may also have adaptive significance in view of the greater heating capacity of the rays having the longer wave-lengths.

The observation that fiddler crabs cease feeding and enter their burrows every 18–24 minutes can be interpreted as a behavioral mechanism for control of body temperature. Every time a crab descends into its burrow the escape from sunlight to the cooler air would allow the body temperature to decrease several degrees.

Palmer (1962), working with a different species of fiddler crab, *Uca pugnax,* found a daily phototactic rhythm; the crabs spent a greater proportion of time in the illuminated end of the apparatus during the morning hours than at other times of day. An attempt at thermoregulation may in part underlie the change in strength of phototaxis because between 5 and 8 AM, when the crabs show a strong positive response to light, the air is usually cool, but during the warmer portion of the day the attraction to light, hence the tendency to leave the burrows, is not so great.

SUMMARY AND CONCLUSIONS

1. Upper thermal death points were determined for the fiddler crab, *Uca pugilator.* In saturated air the lethal temperature for 50% of the crabs, determined

graphically from the experimental data, after an exposure of one hour, was 40.7° C. All of the crabs died after one hour at 42° C. In dry air the corresponding temperatures were 45.1° C. and 47° C. for the same time of exposure.

2. Five minutes after having been placed in sunlight the body temperature of dark crabs was 2° C. higher than that of pale crabs. More visible light is reflected from the dorsal surface of the cephalothorax of a pale crab than from a dark crab. The difference is more striking with the rays of longer wave-length which have a greater heating capacity than the rays at the violet end of the visible spectrum. These observations support the hypothesis that the blanching that occurs at high temperatures has a thermoregulatory role.

3. The body temperatures of crabs maintained either in dry air or in air having a relative humidity of 50% were lower than the air temperature, undoubtedly due to transpiration of water. The body temperatures of crabs in saturated air were the same as the air temperature. The cooling that resulted from transpiration was proportional to the decrease in relative humidity.

4. Transpiration from this crab is a passive process. The rate is proportional to the saturation deficit of the air.

5. In their habitat specimens of *Uca pugilator* exhibit a "feeding-retreat" rhythm that has a frequency of 18–24 minutes. There is no phase interaction between individuals in a population. On hot days the frequent periodic return to the cooler burrows can serve to lower the body temperature.

6. These findings were discussed in relation to the observations of other investigators.

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STAGES IN THE NORMAL DEVELOPMENT OF FUNDULUS HETEROCLITUS[1]

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Fundulus heteroclitus (Walbaum), the mummichog, is found along the Atlantic and Gulf coasts from the Gulf of St. Lawrence to Texas (Bigelow and Schroeder, 1953). It is particularly abundant in the northern part of its range and, according to Nichols and Breder (1927), is replaced on the Gulf Coast by a closely related species. The typical habitats of *F. heteroclitus* include the inshore bays and inlets as well as the shallow tidal creeks, ditches and pools. According to Bigelow and Schroeder, practically all of the mummichogs of the Gulf of Maine will be included within a line drawn 100 yards out from the strand.

The adults commonly measure 2 to 4 inches in length with occasional individuals measuring as much as 6 inches. The adults show a striking sexual dimorphism during the breeding season. The males are blue-black dorsally with a black spot on the caudal aspect of the dorsal fin. Ventrally they are predominantly yellowish. Pale vertical stripes may be present on the trunk and tail. The colors are less intense during the non-breeding season. The females are somber in appearance, olivaceous above, grading to pearly white on the ventral surface. Faint darker vertical stripes are sometimes present, most prominent on the tail. These stripes are usually lacking on the larger females. Both sexes show adaptive changes in color intensity to changes in background.

Fundulus heteroclitus has been used extensively for embryological research due, in large measure, to several advantages. The adults are readily collected in minnow traps or by seining, the eggs may be stripped from the females and fertilized in the laboratory. The chorionic membrane is transparent, affording a clear view of the developing embryo, the embryos are hardy and of adequate size for operative procedures, and the pace of development, closely resembling that of *Amblystoma punctatum,* is convenient for a variety of experimental problems.

The ovarian eggs are small in the fall, ranging from 0.16 to 0.4 mm. in diameter (Eigenmann, 1890). There is little change in size between October and April.

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The eggs increase slowly in size during April and by May 1, the largest eggs measure 0.8 mm. in diameter. According to Eigenmann there is rapid increase in the size of the eggs during May so that they reach their full size by June 1. However, on Cape Cod some fish do mature their eggs as early as the middle of May. Such adults are found in the smaller creeks, the pools and the ditches where the water warms up early in the season. The spawning season ends about the middle of July or shortly thereafter.

Gentle pressure on the abdomen directed posteriorly will express the eggs from a ripe female. The milt can be obtained in a similar manner from the males. Fertilization is carried out conveniently in a glass fingerbowl containing enough water to wet the bottom of the dish. Rocking the bowl gently after the addition of the milt will usually assure the fertilization of the eggs. Difficulty is experienced in stripping if the eggs are immature. Such that are obtained will be white and opaque, in contrast to the mature eggs which are yellow and transparent. Difficulty in expressing the milt from the males can be circumvented by removing and mincing the testes in sea water and then decanting the sperm suspension onto the eggs. Eggs remain fertilizable for 15–20 minutes after stripping (Kagan, 1935). Adults collected in the larger inlets early in the breeding season may not have fully matured their eggs. Such fish, if held in the laboratory, will frequently mature their eggs over a period of days, permitting the collecting of a limited number of eggs daily for as long as eight or ten days. At the height of the season a fully ripened large female may yield several hundred eggs at a single stripping and, under fortunate circumstances, better than 98% of the eggs can be fertilized.

The embryos are hardy. According to Solberg (1938), normal development proceeds at temperatures ranging from 12° to 26° or 27° F. They also develop in a wide range of salinities, even in distilled water, with some minor deviations from the normal. Developing in sea water, the embryos show an increase in the perivitelline space as the yolk is utilized in growth and differentiation. In distilled water the yolk sac, instead of decreasing, increases in size. Eventually the perivitelline space is obliterated, the embryo and yolk sac completely filling the chorion. Contraction of the somitic muscle is lost and the heart shows certain abnormalities of beat conduction with dropped beats in the ventricle. These physiological changes may be related to shifts in the calcium-potassium ratios incident to the onset of kidney function (Armstrong, 1932).

The oxygen requirements of developing *Fundulus* embryos initially are low (Amberson and Armstrong, 1933). The rate of development appears unaffected if the eggs are crowded during this period. However, on the second day the oxygen consumption starts rising sharply so it is essential to avoid crowding or clumping of the eggs in a culture if uniform parallel development of the embryos is desired.

It is necessary to use a variety of lighting methods, at times in different combinations, to reveal the various morphological features of the embryos. The pigment cells show up to best advantage with light from a white substage reflector, the circulation with a substage mirror. Higher intensity of illumination from above, as from a Zirconarc lamp, is required to reveal finer details of structure. There are minor time variations in which structures differentiate in relation to each other so that pinpointing any stage in time can be difficult and, on occasion, may appear somewhat arbitrary. Consecutively numbered stages are figured starting with the ovum, then proceeding with the fertilized egg.

Additional information on techniques and experimental methods for handling *Fundulus* eggs are described by Costello *et al.* (1957). There are references to technical procedures in work done by Nicholas (1927), Oppenheimer (1959) and Trinkaus (1951). Supplementary information on *Fundulus* staging is available in Oppenheimer (1937), and Solberg (1938).

Stage 1 [2]

The fully matured unfertilized ovum is about 2 mm. in diameter. Eggs from the smaller females are commonly somewhat smaller than those from the larger fish. The egg membrane or chorion is 10–12 μ thick (6.5 μ; Eigenmann) and is composed of two layers (Shanklin and Armstrong, 1952), an inner compact meshwork of protein fibrils and an outer more homogeneous layer from which many fine fibrils arise to form a loose matting on the external surface of the membrane. When the ova are shed these fibrils are sticky and adhesive, a property soon lost on exposure to sea water. This membrane with its fibrils has been described by Eigenmann as a product of the follicle cells of the ovary. There are fine pores, 1.0–1.5 μ in diameter, through the external layer of the chorion) numbering 10,000-20,000 mm.^{2}. The micropyle is most readily seen immediately after stripping the eggs from the fish. It shows up best when viewed on the horizon of the egg and appears as a small funnel-shaped indentation on the external surface of the chorion.

The cortical granules (yolk platelets) are uniformly distributed over the surface of the yolk, just deep to the chorion, together with an aggregation of oil droplets in various sizes. There are a few small oil droplets scattered through the yolk which otherwise is a clear yellow viscous fluid, not granular as in some teleost eggs.

Fertilization

Dramatic changes occur following fertilization. The cortical granules disappear, the perivitelline space forms, and the protoplasm heaps up to form a single cell, continuous at its margins with the plasma membrane surrounding the yolk.

In addition, Huver (1960) has described the formation of the polar bodies, based on time-lapse microcinematography. He observed the first polar body approximately three minutes after fertilization. It was closely compressed to the egg surface in freshly stripped eggs. The second polar body appeared 4.5 to 5.0 minutes after fertilization, adjacent to the first.

Immediately following fertilization the cortical granules disappear in a progressive wave spreading out from the micropyle, the process being completed in 2-3 minutes. The "lifting" of the chorionic membrane occurs first in the area encircling the micropyle, developing slowly immediately after fertilization. There is a brief delay in actual separation at the micropyle, this taking place quite regularly at approximately three minutes. It is noted that immediately after complete separation, the subjacent surface area of the egg assumes a dusky complexion characteristic of protoplasmic aggregation. This culminates in the formation of the one-cell stage through further protoplasmic accumulation at this site. In *Fundulus* the chorionic membrane, which functions as a fertilization membrane, does not actually lift but rather the egg shrinks away from the membrane as the perivitelline space forms. This progressive formation of the perivitelline space closely parallels the disappearance of the cortical granules.

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[2] The stage numbers used throughout this paper are illustrated by figures with corresponding numbers, in the plates at the end of the paper.

Mature ova can be activated merely by stripping them into sea water. They go through similar changes as described above for fertilized eggs but not identical in all respects. The perivitelline space forms rather slowly, the cortical granules disappearing in a mosaic progression rather than in a continuous wave. Also, the aggregation of the protoplasm to form the protoplasmic cap is slower.

Stage 2

It is usually stated that the one-cell stage forms as a result of the "streaming" of the protoplasm from its general distribution over the surface of the egg to the submicropylar area. This "streaming" is not visible as such and, in the large egg of *Fundulus,* is a slow process. As noted above, there is some protoplasmic condensation at the submicropylar area when the perivitelline space first forms. At the end of an hour the one-cell stage appears as a biconvex lens-shaped structure. At the end of an hour and a half the protoplasmic cap bulges above the curve of the egg and at one and three-quarters hours the single cell is fully formed as illustrated.

Stage 3

The first division is meroblastic and meridional, resulting in two cells of equal size.

Stage 4

As the second division approaches, the two primary blastomeres flatten down, reducing the depth of the furrow between them. The second division is meridional and at right angles to the first, resulting in four blastomeres of approximately equal size.

Stage 5

The 8-cell stage results by a vertical division of the cells of the four-cell stage, the lines of cleavage paralleling that of the first cleavage. Two parallel rows of four cells each are commonly seen but minor irregularities in this arrangement are not uncommon.

Stage 6

Another vertical cleavage produces 16 cells in a single layer of four parallel rows of four cells each, though again minor variations in cell alignment may occur.

Stage 7

The cells of stage 6 appear columnar as they approach cleavage, particularly the central cells. The cleavage spindles are perpendicular to the egg surface, this cleavage being latitudinal. The central cells continue as a two-celled layer. There is some rearrangement of the peripheral cells as cleavage proceeds, with irregularities in the layering of the cells. In some specimens the yolk appears to jut up into a depression on the deep surface of the blastodisc.

Stages 8–9–10

With successive cleavages there is a progressive increase in the number of cells with a reduction in cell size. However, there is little apparent increase in the size of the blastodisc, marking this early period as one of cell multiplication rather than one of growth.

Stage 11

The blastodisc is flattening out over the yolk (Trinkaus, 1963). The peripheral margin is serrated as if the periblast is about to migrate out. If the blastocoele has formed, it is not visible in the living embryo.

Stage 12

The blastodisc is circular as seen from above and still has a well defined border. However, with proper illumination, the peripheral or marginal periblast nuclei can be visualized on the surface of the yolk at the margin of the blastodisc, forming a circumferential band about one-fourth the diameter of the blastodisc. The nuclei are disposed in four to five irregular concentric rows without cell boundaries. The density of the associated protoplasm can be demonstrated by staining with neutral red or by fixing the material in Stockard's solution (substitute 0.7% NaCl for distilled water to avoid shrinkage). This protoplasmic condensation and the periblast nuclei are not depicted in the illustrations. The former is seen only after fixation or vital staining. The periblast nuclei are not visible at the magnifications used for the illustrations, but are visible at higher magnifications, if the egg is illuminated with daylight both from above with direct light and from below with a substage mirror. The blastodisc appears as a biconvex disc when viewed on the horizon of the egg, the outer or free surface being of greater convexity than the surface in contact with the yolk. The blastocoele cannot yet be discerned in the intact egg.

Stage 13

There has been some additional flattening and expansion of the blastoderm over the yolk. The blastocoele may be seen indistinctly in the transilluminated egg if the blastodisc is viewed on the horizon of the egg. The peripheral periblast is still relatively wide, the nuclei being somewhat irregularly distributed, three to four nuclei in width. Fine droplets are aggregating at the ~~animal~~ vegetal pole, marking the future site of the closure of the blastopore.

Stage 14

There has been a further extension of the blastoderm over the yolk and an elevation of the central area of the blastoderm, with an increase in the blastocoele resulting in a wide separation of the epiblast from the hypoblast. The peripheral periblast is somewhat reduced in width as the blastoderm has expanded. This stage may mark the onset of the transition from the blastula to the gastrula.

Stage 15

There is a definitive germ ring in this stage which is narrow but complete around the margin of the blastoderm. The embryonic shield is rudimentary, slightly wider than the rest of the germ ring and slightly more elevated when viewed on the horizon of the egg. The early embryonic shield frequently overlies the oil droplets. The peripheral periblast is narrow, reduced to a few nuclei at the immediate margin of the germ ring. The blastocoele is extensive under the extra-embryonic ectoderm, separating the epiblast rather widely from the hypoblast, especially just anterior to the embryonic shield. The blastocoele is not necessarily bilaterally symmetrical in its form.

Stage 16

As epiboly proceeds, the blastoderm extends over the surface of the yolk, the germ ring advancing ahead of the extra-embryonic membrane. Concurrently the embryonic shield increases in size, the embryonic axis in length. There is a further accumulation of fine droplets at the vegetal pole at the future site of the closure of the blastopore.

Stage 17

The gastrula has expanded to cover one-half of the yolk. The germ ring is little if any wider but the embryonic shield and axis have extended with epiboly. The latter is about one-sixth the circumference of the egg in length. It is best seen when viewed on the horizon of the egg. The fine droplets on the vegetal pole are converging to form a closer aggregation.

Stage 18

The extra-embryonic ectoderm now covers three-fourths of the surface of the yolk. The embryonic shield has extended in length and the embryonic axis can be clearly discerned not only when viewed on the horizon of the egg but also in a direct dorsal view.

Stage 19

The blastopore is reduced to a small opening through which the yolk may bulge. The embryonic axis is well defined with some condensation of tissue along its lateral margins. The optic vesicles are present but rudimentary. The embryonic keel is prominent in this stage.

Stage 20

The closure of the blastopore is complete at this stage. The main divisions of the brain, the forebrain, midbrain, and hindbrain, are distinguishable with a well defined keel, ventral to the brain, indenting the yolk sac. There is an increased but variable condensation of cells lateral to the embryonic axis in the location of the future anterior somites. Kupffer's vesicle appears as the embryos advance toward the next stage. The ectoderm lifts to form a large vesicle anterior to the head, possibly the forerunner of the pericardial cavity.

Stage 21

There are three to four pairs of somites at this stage. The embryo has increased in size, particularly toward the caudal end.

Stage 22

The main divisions of the brain are now more sharply differentiated. There is an impression of segmentation of the hind brain into neuromeres, but the brain ventricles are still unformed. The optic lobes are more prominent than in the preceding stage. The optic cup can be seen best in a lateral view but the lens is not discernible. The pericardial cavity is present anterior to the head but has not yet extended posteriorly under or lateral to the head which is flush with the yolk. There is some condensation of tissue at the future site of the pectoral fin. There are no pigment cells either on the embryo or yolk sac but the blood islands are forming as fine strands on the surface of the yolk. The Kupffer vesicle is deep and anterior to the tip of the tail, which is flush with the surface of the yolk. Under the tip of the tail is a collection of fine droplets.

Stage 23

With additional growth and differentiation, the main divisions of the brain are better defined as are also the otic vesicles. The lens appears as a thickening but does not bulge out of the optic cup. The earliest indication of the olfactory placode can be noted. The outlines of the pericardial cavity, bilateral to the midbrain and hindbrain, are well defined. This cavity extends under the head, which has lifted off of the yolk sac beneath the optic lobes and anterior hindbrain. The anterior extremity of the forebrain is still flush with the yolk sac. In most specimens, the heart rudiment can be seen. There has also been a further elaboration of blood islands on the yolk sac. Pigment cells are present, scattered sparsely over the yolk sac with occasional cells on the dorsolateral aspect of the hindbrain. These cells are small and contain a small amount of densely aggregated pigment. The tip of the tail is rounded but not free of the yolk sac.

Stage 24

The brain ventricles are forming as the embryos approach this stage, initially in the optic lobes with rapid extension into the other divisions of the brain. Some of the neural derivatives also are more prominent, including the olfactory placode and the otic vesicle. The lens of the eye fills the optic cup. There has appeared a condensation of tissue on the lateral sides of the hindbrain both anterior and posterior to the otic vesicles. Also, there is an increased number of melanophores and, as the embryos develop toward the next step, erythrophores appear on the deep side of the embryos under the hindbrain and trunk. Most of the melanophores are still unexpanded. The tip of the tail is rounded but bound down to the yolk sac. The earliest cardiac contractions were observed in embryos in the 14-somite stage and, as the heart elongates, there appears a regular initiation and conduction of pulsations from the venous to the arterial end. The blood islands of the yolk are linking up with each other, forming a syncitium, but there is, as yet, no circulation. Also, the melanophores on the yolk sac have increased in numbers.

Stage 25

This period is marked by the onset of the circulation. The pericardial cavity extends forward, lifting the head off the yolk; the venous end of the heart likewise extends forward to form an elongate tube. Also, there develops a concentration of blood cells in the blood islands on the yolk at the venous end of the heart and near the root of the tail. The circulation is established first through the posterior vitelline arteries and was observed in 16 of 20 embryos in the 19-somite stage. Initially the circulation is sluggish, with red blood cells in scattered clumps moving slowly through the vessels. Late in this stage the circulation is established through the anterior vitelline arteries. The first contractions of somitic muscle are seen in embryos in this stage, involving only a few of the most anterior somites. These contractions and the accompanying relaxations are slow and produce a slight bending of the body axis. The tip of the tail is now free of any yolk sac attachment. There has been some overall increase in the sizes of various embryonic structures but only partial expansion of some of the melanophores which also have increased in number and extended onto the optic lobes.

Stage 26

There has been an increased extension of the pericardial cavity and an elongation of the heart anterior to the head, fully exposing the sinus venosus region of the heart to observation from above. The heart at its arterial end is curved, foreshadowing the development of the ventricle as a defined chamber. Although the heart chambers are not morphologically differentiated they are physiologically differentiated, showing stepwise depression of contractility progressively involving the presumptive ventricle, atrium and sinus venosus if the embryos are immersed in KCl isosmotic with sea water. The brain ventricles have increased in size, especially the fourth ventricle which has a thin roof and is bounded anteriorly by a well defined rhombic lip. The paired otoliths are first observed in this stage as aggregations of very fine dark granules. The tail has increased in length, has lifted off the yolk sac at its root, and the fine droplets at the root of the tail are scattered along the posterior vitelline vessels. At the root of the tail the aorta arches ventrally onto the yolk sac, there branching into the posterior vitelline vessels. Within this arch is some condensation of cells from which the bilobed urinary bladder will form. There are also a few scattered contracted erythrophores on the yolk sac. Somitic movements are more frequent than in the preceding stage but not much more vigorous.

Stage 27

This and the subsequent stages are in general characterized by a continuing increase in the size of the embryo, a gradual decrease in the size of the yolk sac and active organodifferentiation.

The ventricle of the heart forms a definite chamber but not as well defined as later on. The otoliths are dense concrete bodies. There is a bilateral condensation of cells just posterior to the emerging anterior vitelline arteries, the rudiments of the pectoral fins. They may jut up slightly above the yolk sac when viewed on the horizon of the egg along the embryonic axis as the embryo advances through this stage. The body cavity is forming bilaterally but the gut rests ventrally on the yolk sac bisecting the body cavity longitudinally. The tail has increased in length and is still further elevated off the yolk. The melanophores on the surface of the yolk sac are expanding. Body movements have increased in frequency. Also in this stage the pronephros actively eliminates dyes, though the urinary bladder is not yet formed (Armstrong, 1932).

Stage 28

This stage is marked by the earliest development of retinal pigment imparting a dusky tone to the eye. The body cavity has extended under the trunk, usually lifting the gut off the yolk sac. There is a blood vessel coursing caudally along the gut. The urinary bladder is small, bilobed but contains no precipitate. The tail has grown in length and lifted further off the yolk sac but contractility of the somites is limited to those anterior to the ventral flexure of the tail. The pectoral fin juts up above the surface of the yolk sac parallel to the body axis.

Stage 29

There has been an increase in the pigmentation of the eye, which has also increased in size so that the lens does not completely fill the optic cup. The pectoral fin is a small acuminate projection on the horizon of the egg as viewed along the body axis. The urinary bladder is a small bilobed organ grooved above and anteriorly by a ventrally coursing blood vessel. There is no precipitate in the urinary bladder but selected dyes injected into the embryos in this stage are eliminated by the pronephros and color the bladder contents. The ventricle is well defined as a definitive chamber but the demarcation between the atrium and sinus venosus is vague.

Stage 30

Although the retinal pigment has markedly increased, the outline of the lens can still be discerned on transillumination of the eye. The pectoral fin extends slightly above the lateral line. The tail is completely straightened out. On lateral flexion, the tip of the tail passes over the hindbrain. The aorta terminates in a small glomus in the base of the rudimentary caudal fin which is just beginning to flatten out.

Stage 31

The heart chambers are all differentiated. The atrium is to the left and anterior to the ventricle, not directly to the left as it will be later, nor is it yet completely filled out. The tail fin shows additional flattening with a bifurcation of the aorta, one branch extending dorsally into the caudal fin, the other ventrally. The body cavity is well formed with the gut and liver in view. The liver is to the left of the body axis when viewed from above and blood can be seen circulating through the liver sinusoids. A small vessel is forming parallel to the pectoral fin margin. Circulation in this vessel usually appears late in this stage. There is a finely divided precipitate in the urinary bladder, which is bilobed, the two lobes communicating with each other through a large opening.

Stage 32

The retina is more heavily pigmented, so much so that the outline of the lens can be seen only with strong transillumination. The atrium has increased in size. The posterior margin of the operculum is forming below the anterior margin of the otocyst, with a shallow depression extending forward deep to this margin. Branchial arches may be seen. There is a single blood vessel arching in the pectoral fin parallel to its border but as yet no motility is observed in the fin. There is a tripartite branching of the aorta into the caudal fin with additional radial vessels appearing later in this stage. Reflex vagal inhibition of the heart is elicited on mechanical stimulation of the embryo in this stage or shortly thereafter (Armstrong, 1931).

Stage 33

During the past several stages there has been a developing flexure of the head at the level of the hindbrain, which is most marked at this stage. In the succeeding stages the head gradually straightens out. The ectoderm of the yolk sac comes off the embryo anteriorly at the lower level of the forebrain. The rudiment of the lower jaw is inferior to this, under the head. The retina is heavily pigmented, masking the deep part of the lens. There may be sporadic movements of the fins but no coordinated rhythmic movements. Rays in the caudal fin are faintly seen, with some unexpanded pigment cells along the rays. Three to five radial vessels originate from an arterial arch in the base of the caudal fin and pass distally parallel to the fin rays.

Stage 34

The lower jaw is well developed, the mouth is open and occasionally lower jaw movements are observed but these movements are not coordinated with movements of the gills, which are still immobile. The yolk sac ectoderm comes off the lower jaw just below the mouth. The pectoral fins may occasionally show fluttering movements between long periods of quiescence. Body movements evoked by gross stimulation are spasmodic and uncoordinated and are frequently accompanied by fin movements (Coghill, 1933). The rays in the caudal fin are well developed, with blood vessels radiating out between the rays. There is a ventral fin along the entire length of the tail. The swim bladder is small and inconspicuous. Hatching takes place shortly after the mouth has come open, due in part to an enzyme liberated by cells in the mouth and gill cavities (Armstrong, 1936).

Stage 35

Some extension of the head has taken place. The ectoderm of the yolk sac now comes off the ventral aspect of the head just in front of the continuity of the conus arteriosus with the ventral aorta. The margins of the operculum are well defined; no movements were noted but there were movements of the eyes. Undulatory swimming movements occur but are transitory and are not sustained enough to propel the embryo; however, there are well sustained alternating movements of the pectoral fins with intervals of quiescence. A dorsal fin is forming but is not as extensive as the ventral fin.

Stage 36

There has been some further extension of the head and an apparent decrease in the size of the yolk sac. There is little if any significant change in the functional features of these embryos as compared with the preceding stage.

Stage 37

The reduced size of the yolk sac, the extent of the development of the dorsal and ventral fins and the serrations of the caudal fin are aids in differentiating this stage. This stage is also marked by the maturation of motor activity. There is coordination of the lower jaw and opercular movements, and of the undulating swimming and pectoral fin movements.

Stage 38

There is a further reduction in the yolk and a strengthening of swimming activity.

Stage 39

This is a transition stage from the embryonic to the larval state with the complete absorption of the yolk. The operculum and pectoral fins are almost continuously rhythmically active. The swim bladder has increased in size and the embryos commonly swim at the surface of the water but will swim to the bottom of the aquarium if disturbed, the pectoral fins being in constant motion. If the fish are anesthetized, the pectoral fin movements cease and the embryos float to the surface.

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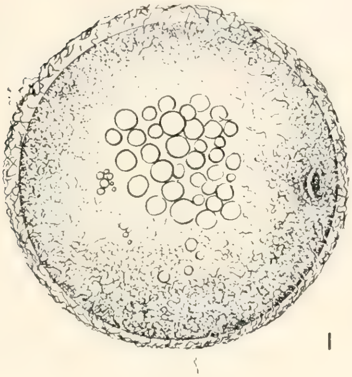
Characterization of Stages

Stage 1	Unfertilized ovum
Stages 2–7	Cleavage
Stages 8–10	Cell multiplication
Stages 11–14	Blastulae
Stages 15–18	Gastrulae
Stages 19–20	Neurulae
Stages 21–39	Growth and organodifferentiation

(Magnification, 22 ×)

Time Sequence of Development in Hours
At 20° C. ± 0.2

Stage	Time	Stage	Time
1	0	21	52:00
2	1:75	22	56:00
3	2:50	23	66:00
4	3:25	24	74:00
5	4:25	25	84:00
6	5:00	26	92:00
7	6:00	27	112
8	7:50	28	128
9	9:00	29	144
10	10:00	30	156
11	11:00	31	168
12	15:00	32	192
13	20:00	33	216
14	24:00	34	228
15	27:00	35	252
16	30:00	36	288
17	33:00	37	336
18	37:00	38	360
19	40:00	39	384
20	46:00		



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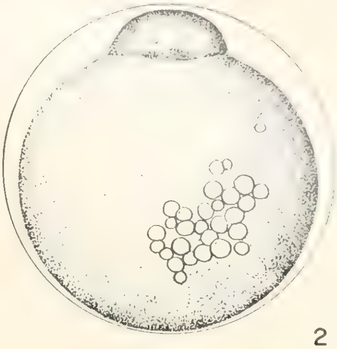

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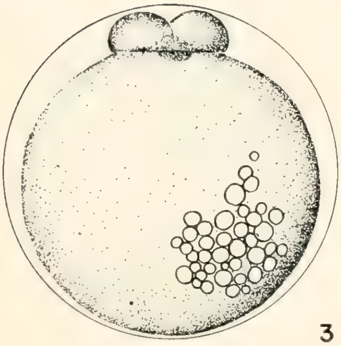

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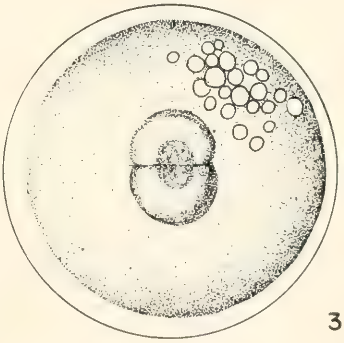

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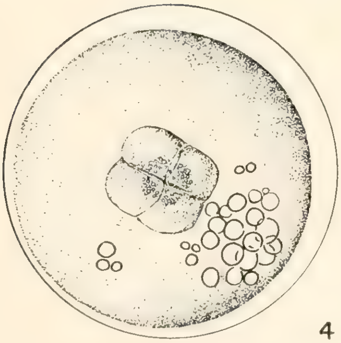

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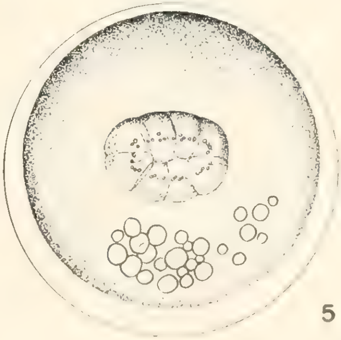

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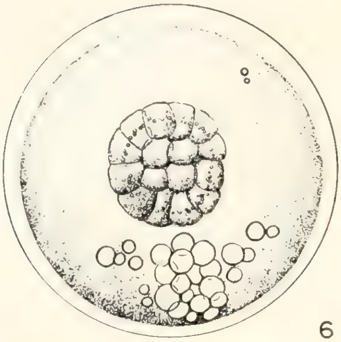

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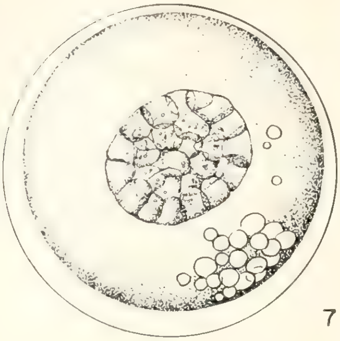

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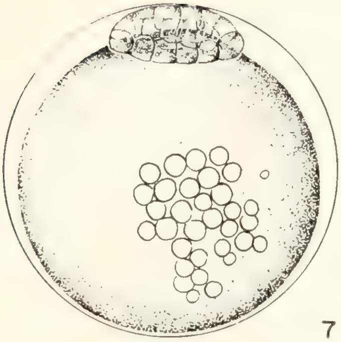

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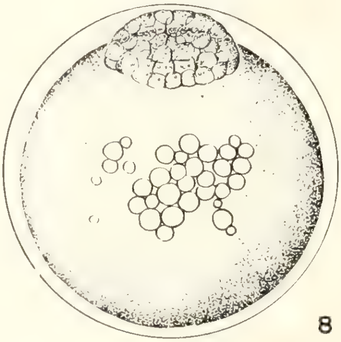

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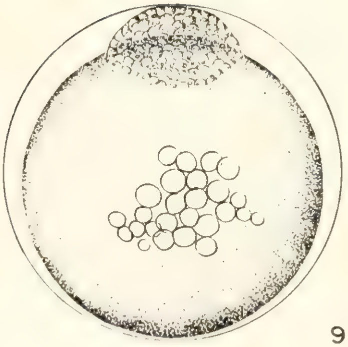

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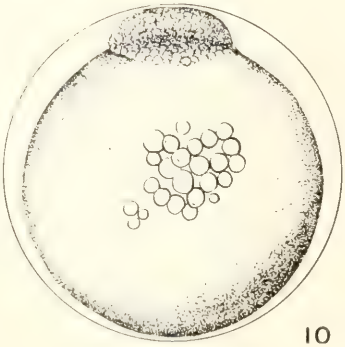

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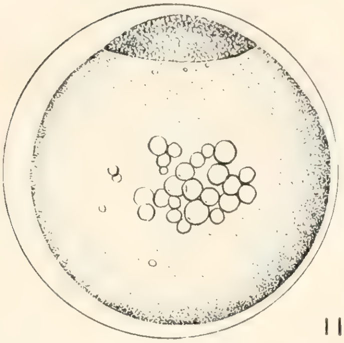

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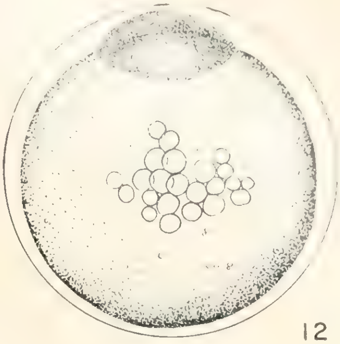

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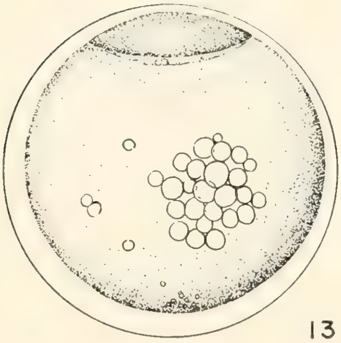

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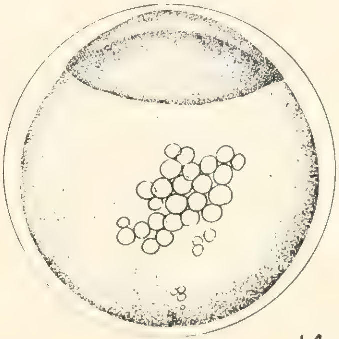

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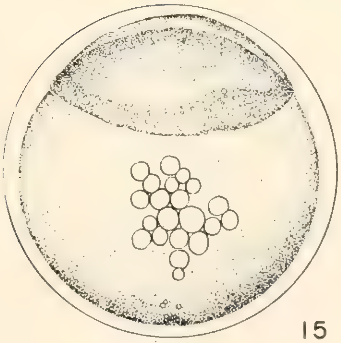

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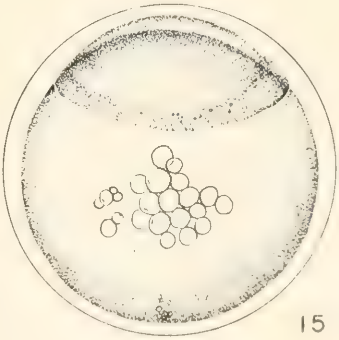

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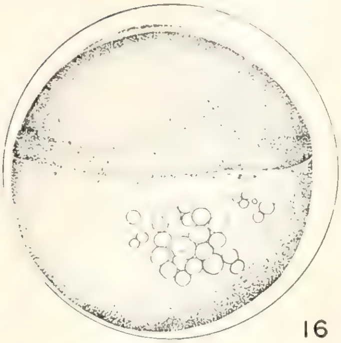

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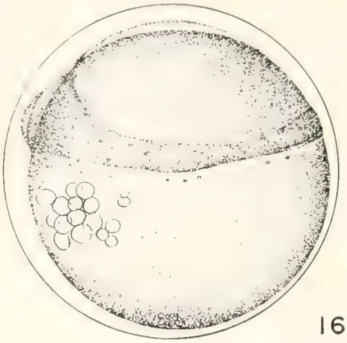

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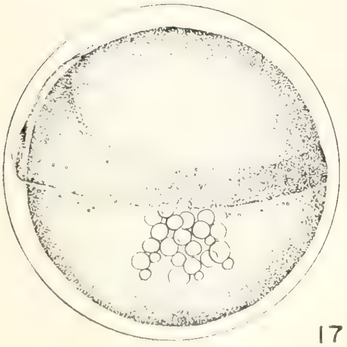

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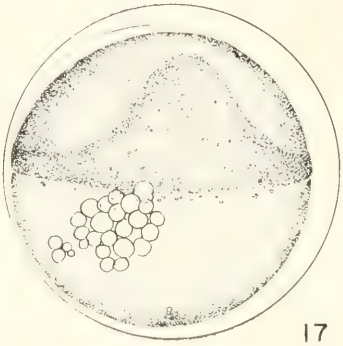

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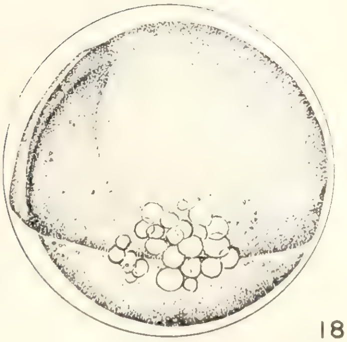

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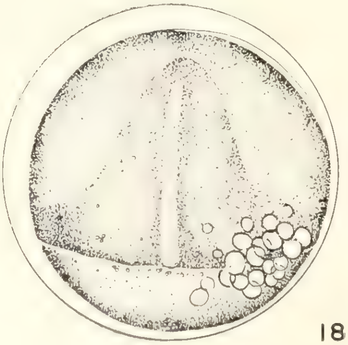

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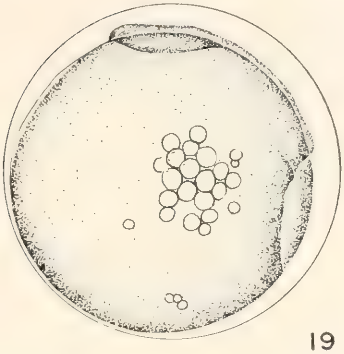

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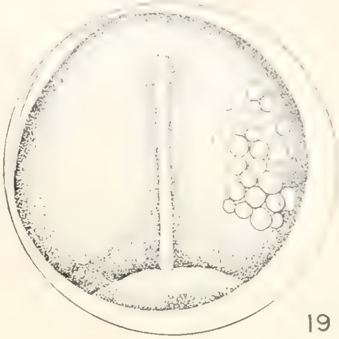

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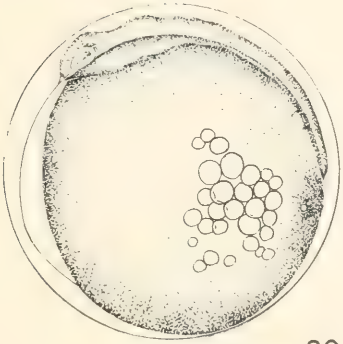

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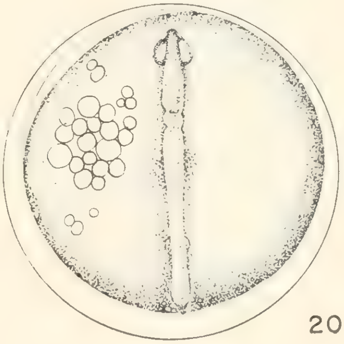

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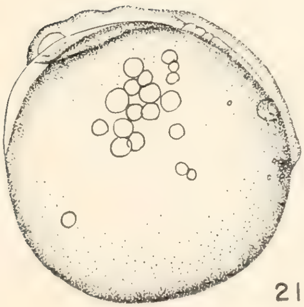

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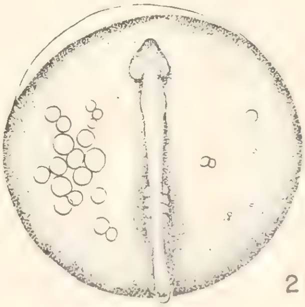

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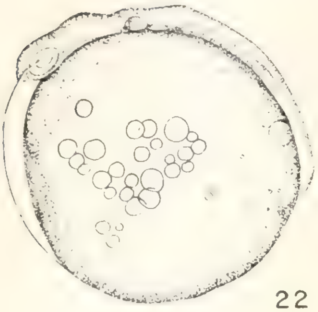

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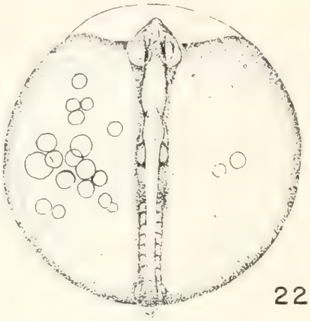

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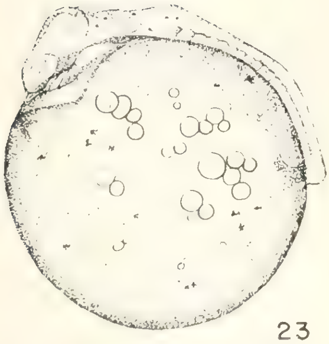

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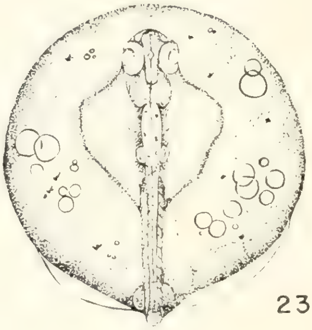

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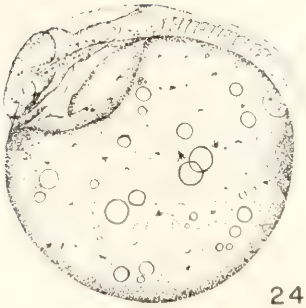

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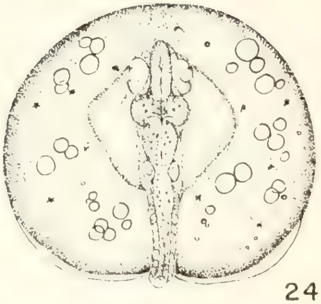

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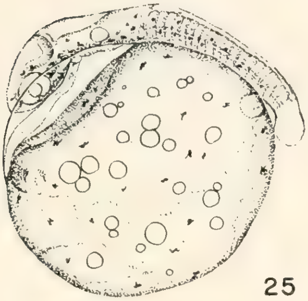
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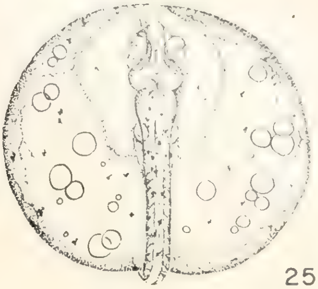
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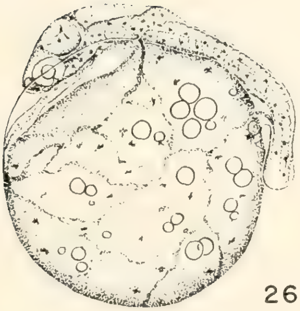
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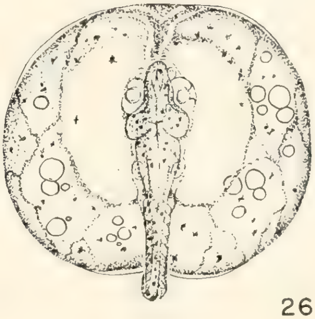
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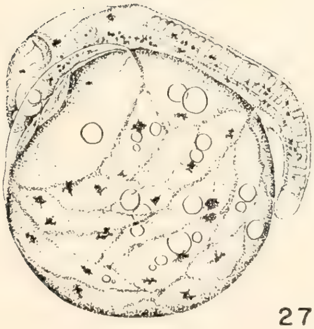
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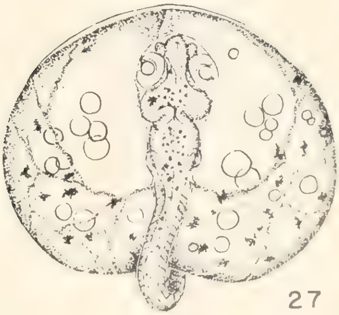
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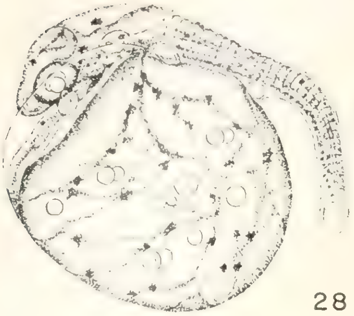

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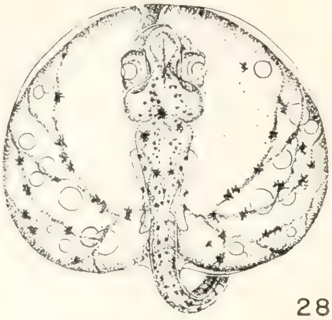

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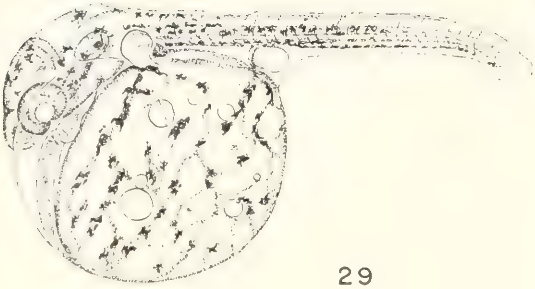

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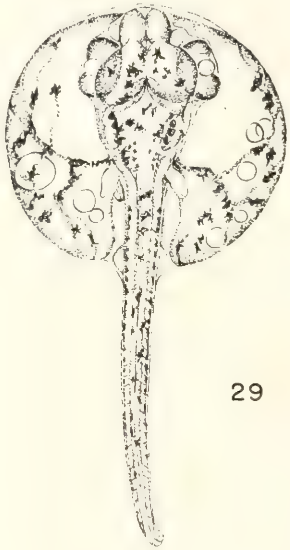

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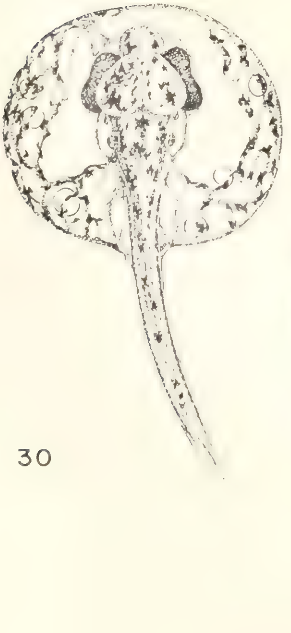

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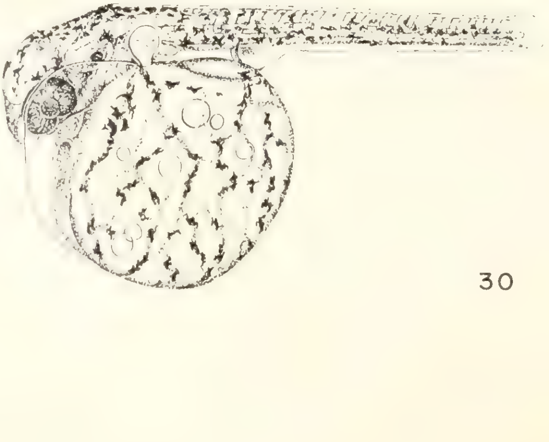

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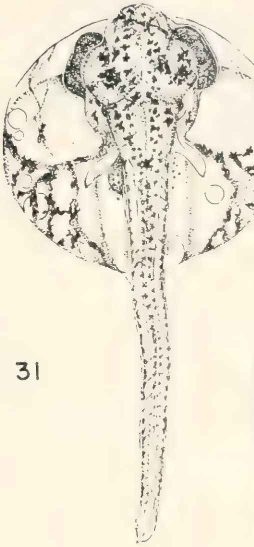

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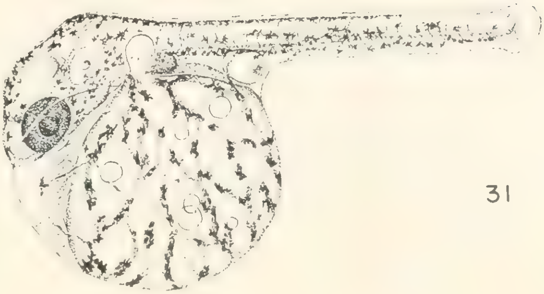

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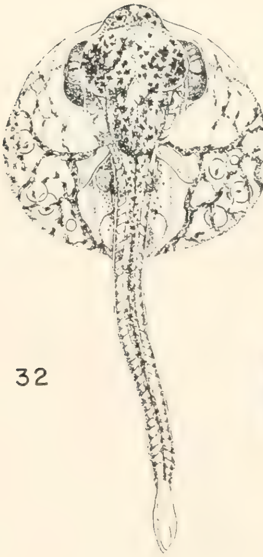

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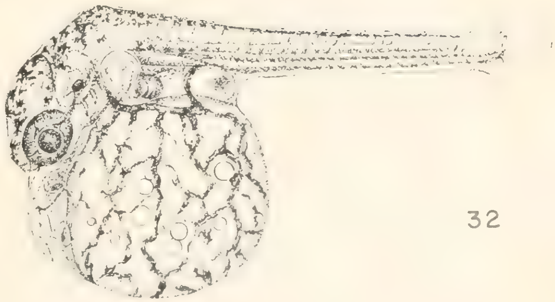

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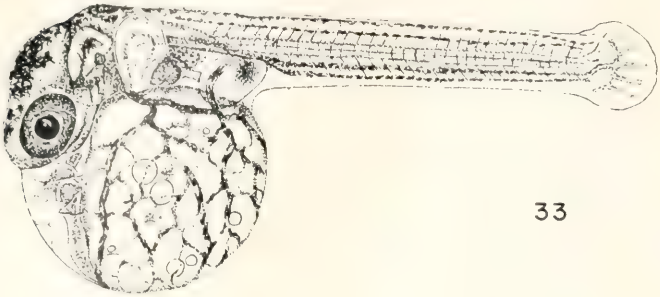

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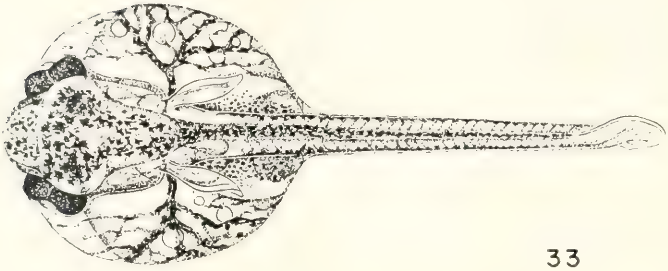

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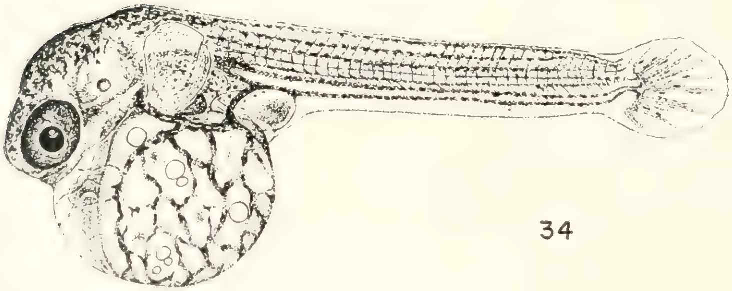

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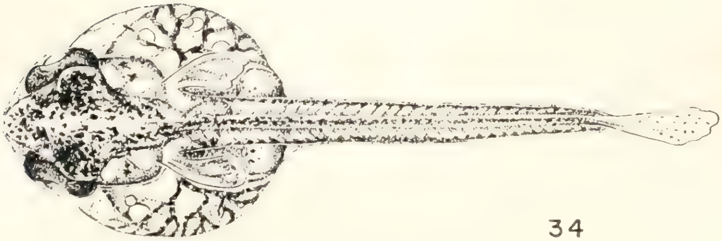

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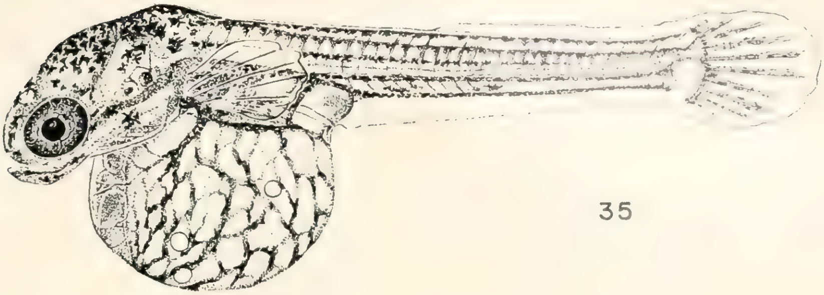

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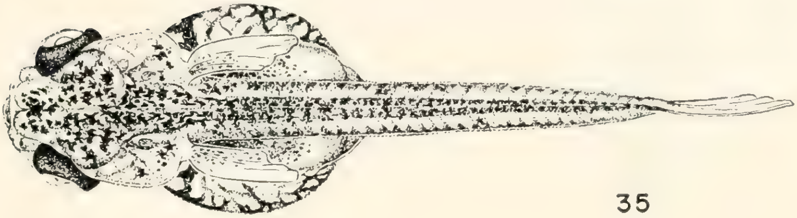

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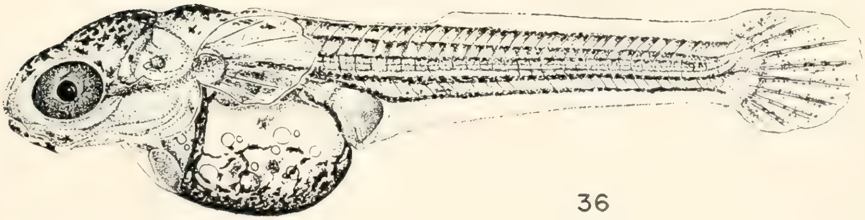

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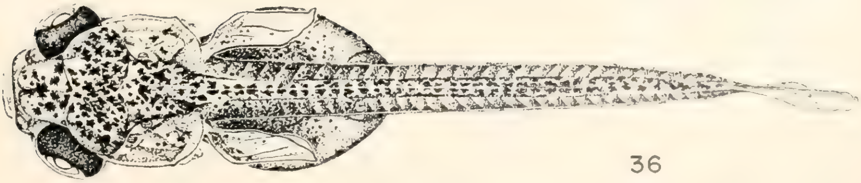

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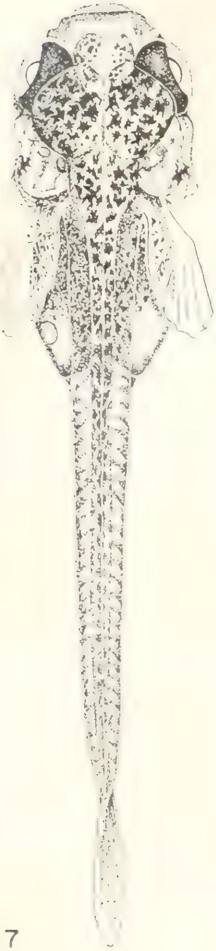

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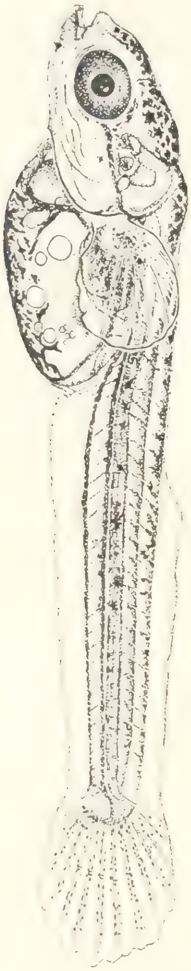

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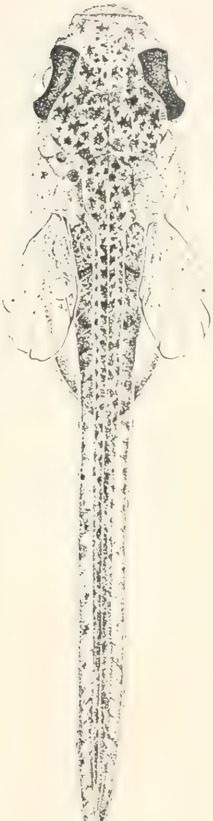

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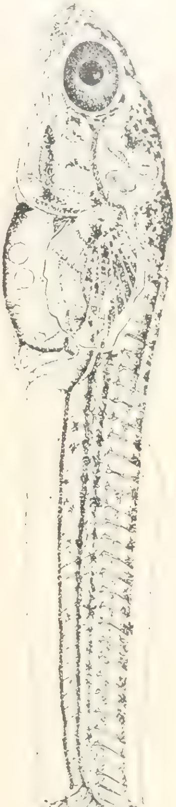

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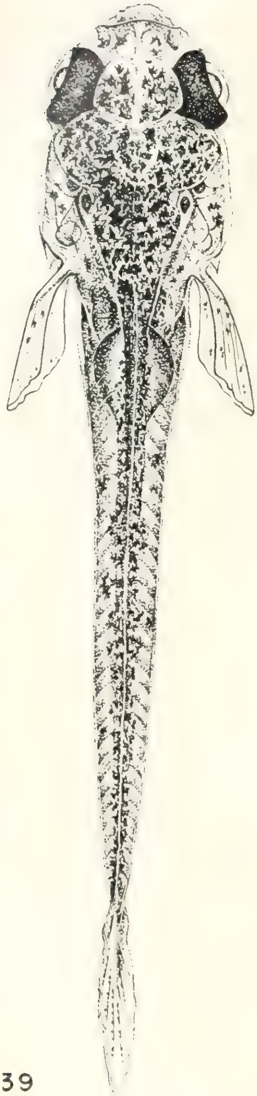

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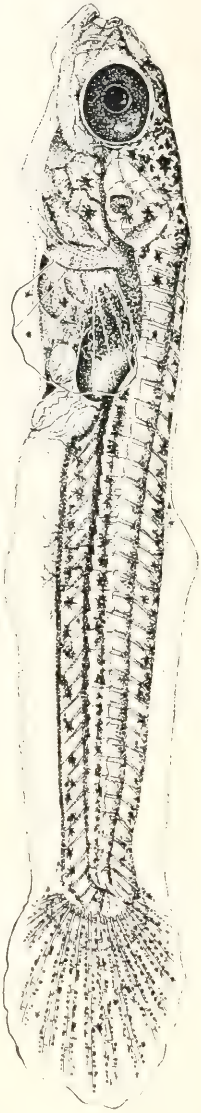

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THE CHROMOSOMES OF SEA URCHINS, ESPECIALLY ARBACIA PUNCTULATA; A METHOD FOR STUDYING UNSECTIONED EGGS AT FIRST CLEAVAGE[1]

WALTER AUCLAIR

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Previously, there has been no truly adequate method for studying the chromosomes of sea urchin eggs, even when sectioning procedures were utilized. With classical methods, such as acetic orcein, carmine and the Feulgen techniques, the cytoplasm stains rather intensely, which tends to obscure the chromosomes. Moreover, the chromosomes are very small and numerous. Consequently, the chromosome number and morphology have never been determined with certainty in *Arbacia punctulata* and in many other marine invertebrates that have large, yolky eggs.

The present paper describes a simple and relatively rapid procedure that has been developed to examine the chromosomes and nuclear events of the early cleavage divisions of unsectioned eggs by a selective staining of the chromatin. With this method the chromosome number of *A. punctulata* has been established and that of the sand dollar, *Echinarachnius parma,* closely approximated. Also included is a description of the morphology of the chromosomes of the former species. A preliminary report has been published previously (Auclair, 1964).

THE METHOD

Shedding of the eggs of both *A. punctulata* and *E. parma* was induced by injection of 0.5 ml. of 0.5 *M* KCl solution into the peritoneal cavity (Harvey, 1956). Dry sperm preparations were prepared by removing the testes and storing them at 4° C. in a dry stender dish.

For optimal results the fertilization membranes were removed. Exactly two minutes following insemination, mercaptoethylgluconamide was dissolved in the egg suspension to a final concentration of approximately 10^{-3} *M* (Harris, 1961). The loosened membranes were stripped off 15 minutes later by straining the eggs through a 25-mesh silk cloth. To prepare the eggs for metaphase chromosome spreads, colchicine was added about 20 minutes prior to the first cleavage division, followed by hypotonic treatment in 50% sea water 5 minutes before fixation.

The eggs were fixed in 50% ethanol at 4° C. for a minimum of one-half hour. Actually, any dehydrating agent, such as acetone, may be used. The specimens may be stored in the fixative for several weeks without any apparent impairment of staining quality.

For the staining procedure the fixative was decanted and replaced directly,

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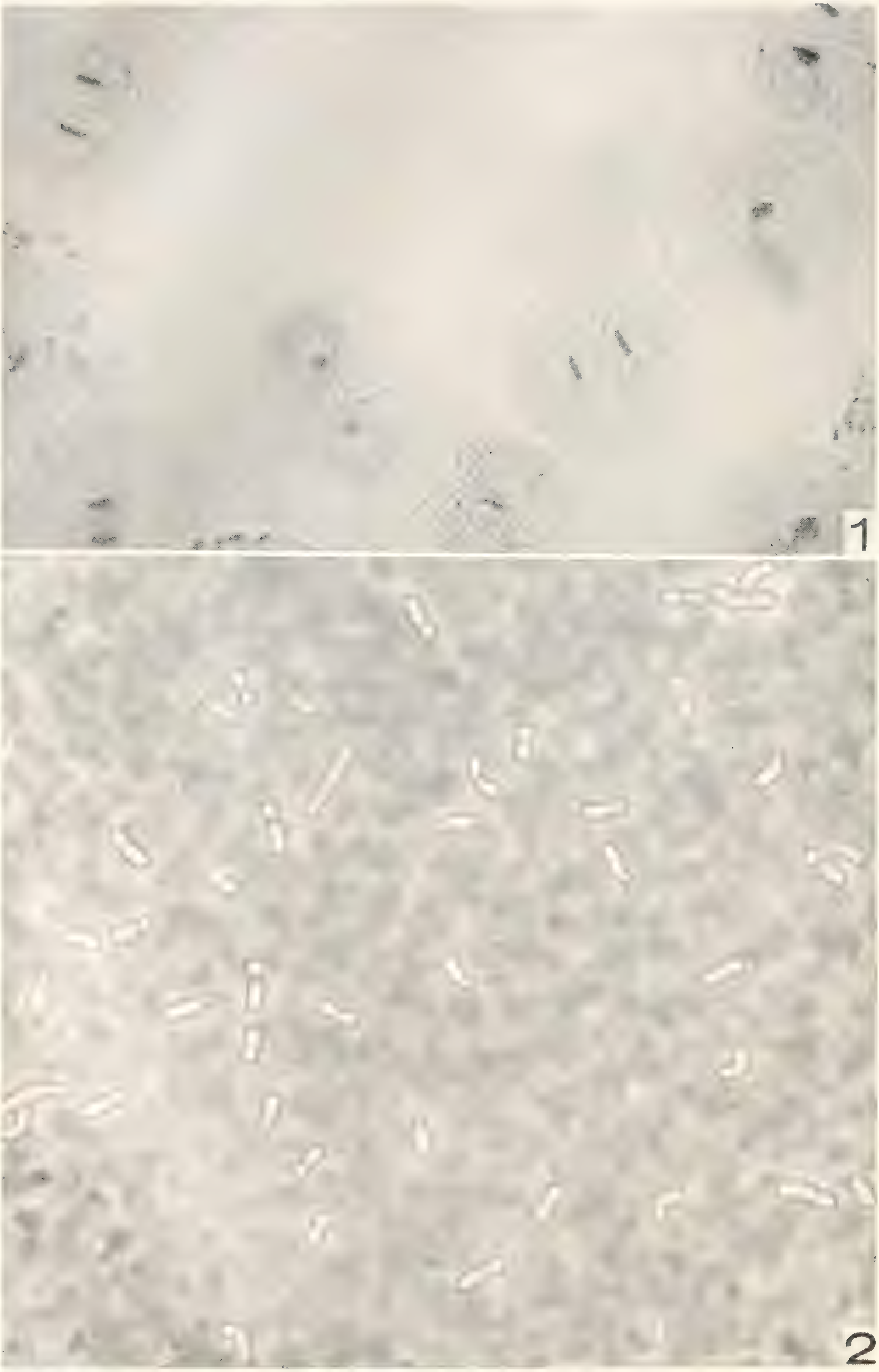
[1] Supported in part by U.S.P.H.S. Grant CA 6439.



FIGURES 1-2.

without rinsing in distilled water, with a solution made by dissolving 0.1 gm. of adenosine 5′-monophosphate (AMP) in 20 ml. of 0.2 *M* Tris-maleate-NaOH buffer at a pH of 5.0 to 5.5. A number of different phosphates were used at an equivalent concentration, but the best results were obtained with organic phosphates, particularly AMP, that are not hydrolyzed to any extent by cellular phosphatases. To the buffer-organic phosphate mixture was slowly added 1 ml. of [illegible] $Pb(NO_3)_2$. The fine lead hydroxide precipitate that often appeared was removed by either filtration or centrifugation. The pH of the medium controls the speed of the reaction; the lower the pH the more rapid is the reaction, but background cytoplasmic staining is also increased at low pH. Incubation time has to be determined for each species. For *Arbacia* eggs, the optimal time for exposure to the medium was 1 to 1½ hours at 37° C., whereas for the sand dollar eggs, which are about twice as large as *Arbacia* eggs, several hours were required.

The eggs were then rinsed thoroughly with several changes of distilled water to remove excess lead ions, and then placed in 50% ammonium sulfide for several minutes. Following another rinsing the eggs were mounted in a water-soluble mounting medium or squashed in distilled water, making spread preparations of the chromosomes.

Nature of the Staining Reaction

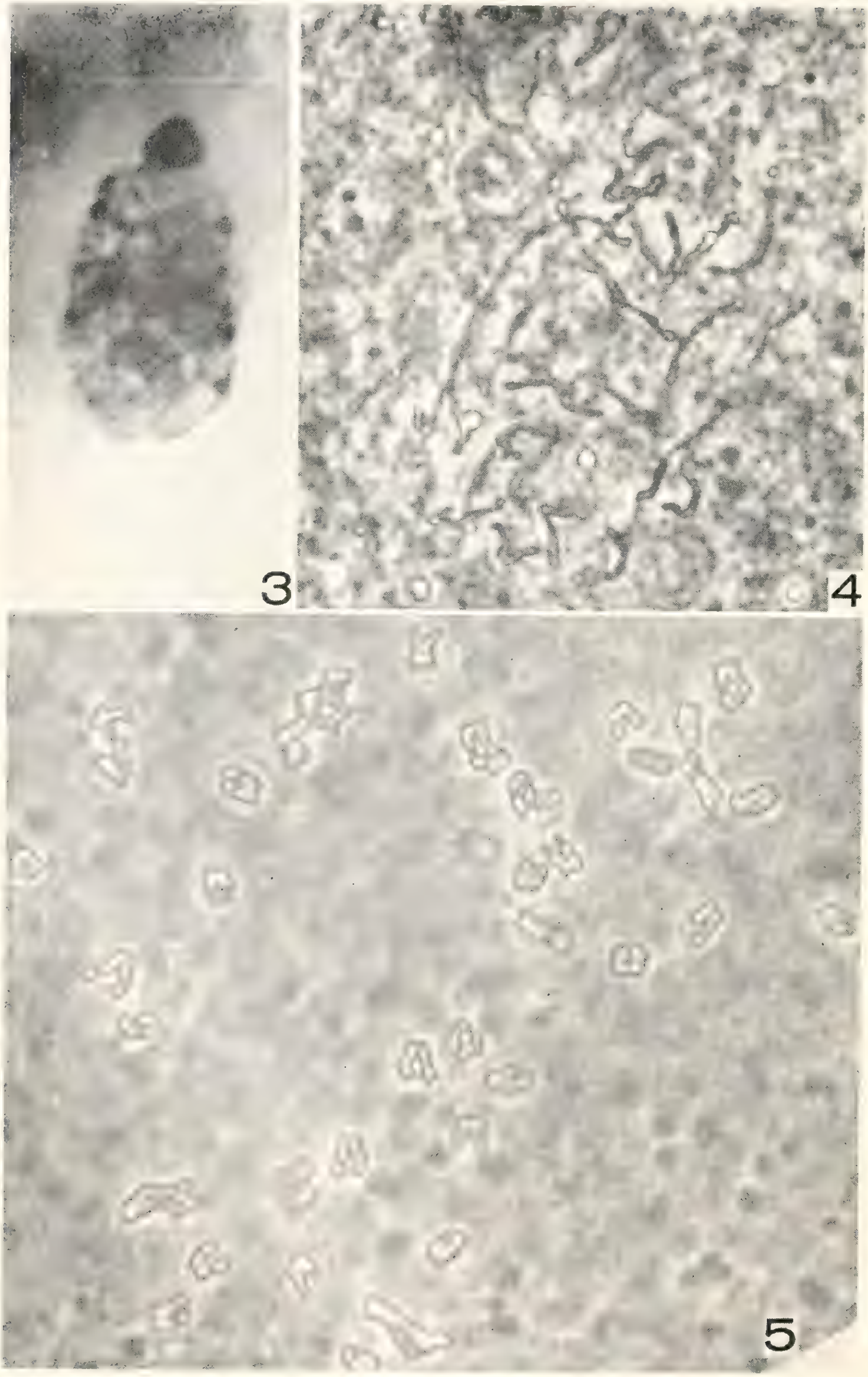
The staining reaction is a modification of the Gomori phosphatase technique, except that enzymatic activity is absent and only the nuclear structures are demonstrated. No chromosome staining occurred if either the lead or organic phosphate was absent. Any possibility of enzymatic hydrolysis was eliminated by the fact that when inhibitors of 5′-nucleotidase or acid phosphatase, 0.01 *M* $Ni(Cl)_2$ or 0.01 *M* NaF, respectively, were added to the lead-AMP solution, the staining intensity was not reduced. The reaction, moreover, is not specific for polymerized DNA. Digestion of the eggs in deoxyribonuclease (0.2 mg. per ml. of either 0.2 *M* Tris-maleate or phosphate buffer at pH 7.2 for four hours at 37° C.) resulted merely in a slight weakening of the staining intensity.

Further insight into the staining mechanism is provided by the following experiment. Ethanol-fixed eggs were placed for one hour (37° C.) in separate solutions of 0.2 *M* Tris-maleate buffer at pH 5.0 containing either 0.05% $Pb(NO_3)_2$ or 0.01 *M* AMP. Following a brief rinsing in distilled water, the lead-treated eggs were placed in buffer-AMP medium, and the AMP-exposed eggs were transferred to the buffer-lead solution. Only those eggs first exposed to the lead and then to the organic phosphate media gave a positive reaction. Inorganic phosphate used in place of AMP gave negative results in every case.

On the basis of these tests it seems probable that the lead ions diffuse into the partially dehydrated chromatin material and attach by ionic bondings to phosphate and other negatively charged sites. Organic phosphates, such as AMP, also diffuse inward and bind ionically with both lead and basic protein, thus anchoring the lead and reducing its susceptibility to removal by subsequent rinses. The sulfide

FIGURE 1. A general view of unsectioned *Arbacia* eggs in various stages of mitosis. Such preparations may then be squashed to make chromosome spreads. Magnification, approximately 400 ×.

FIGURE 2. Pre-metaphase chromosome spread of a colchicine-treated *Arbacia* egg in the first cleavage division. Oil immersion-phase contrast; magnification, approximately 2000 ×.



FIGURES 3–5.

ions then replace the phosphate groups from the lead ions, resulting in the precipitation and deposit of black lead sulfide at these sites. Thus, the term "staining" is not used in an exact sense, since no true dyeing process occurs.

NUCLEAR AND CHROMOSOMAL OBSERVATIONS

By this method the sequence of nuclear events from fertilization through the first two cleavage divisions has been examined in unsectioned material. Background staining tends to be greater in the cytoplasm prior to the formation of the mitotic figure of the first cleavage division.

Syngamy and early prophase. The process of syngamy is revealed in striking fashion. The chromatin of the male pronucleus undergoes marked condensation into very densely staining structures as the two pronuclei come into contact (Fig. 3). Then the male pronucleus flattens itself against the contour of the female pronucleus and gradually the nuclei undergo fusion.

In early prophase the extended chromatid fibers may be seen coiling into elongated chromosomes (Fig. 4). The chromosomes then gradually shorten until they are 3 to 6 microns in length and 1 micron thick. During this shortening process a centromere constriction develops at some point along the length of each of the chromosomes (Fig. 2).

Chromosome morphology and number. The pre-metaphase chromosomes, prior to the appearance of any discernible lines of division, are relatively easy to analyze (Fig. 2). Later the analysis is more difficult because each chromosome continues to shorten as it divides (Fig. 5). Separation of the chromosomes is not uniform, and there is no characteristic attachment point at the centromeres, as seen in many mammalian preparations. The chromosomes lose the centromere constriction during anaphase as they continue to shorten. Figure 1 demonstrates a number of stained, unsectioned eggs in various stages of mitosis.

Repeated counts on over 200 pre-metaphase chromosome spreads of colchicine-treated eggs during the first cleavage division make it certain that the diploid chromosome number in *Arbacia punctulata* is 44. Moreover, in one experiment in which 5% of the controls showed dispermic cleavage, an examination of the chromosome spreads revealed about the same percentage of eggs that had 66 chromosomes. These triploid counts provide a further confirmation for the diploid count of 44.

The karyotype. Chromosomal length and centromere position provided the morphological criteria by which a karyotype (Fig. 6) was prepared from the chromosome spread of Figure 2. Table I provides a specification of the chromosome pairs into groups based on their morphological similarities.

FIGURE 3. Amphimixis in an unsectioned egg of *Arbacia punctulata*. The smaller, more condensed male pronucleus is coming into contact with the larger female pronucleus. The male pronucleus then flattens over the female pronucleus surface and gradual fusion occurs. Magnification, approximately 2000 ×.

FIGURE 4. A partially squashed prophase preparation of an *Arbacia* egg, demonstrating the coiling of the elongated chromatid threads. Oil immersion-phase contrast; magnification, approximately 2000 ×.

FIGURE 5. Metaphase chromosome spread of a colchicine-treated *Arbacia* egg in the first cleavage division. The chromosomes do not split in synchrony and there is no attachment at the centromere position. Oil immersion-phase contrast; magnification, approximately 2000 ×.

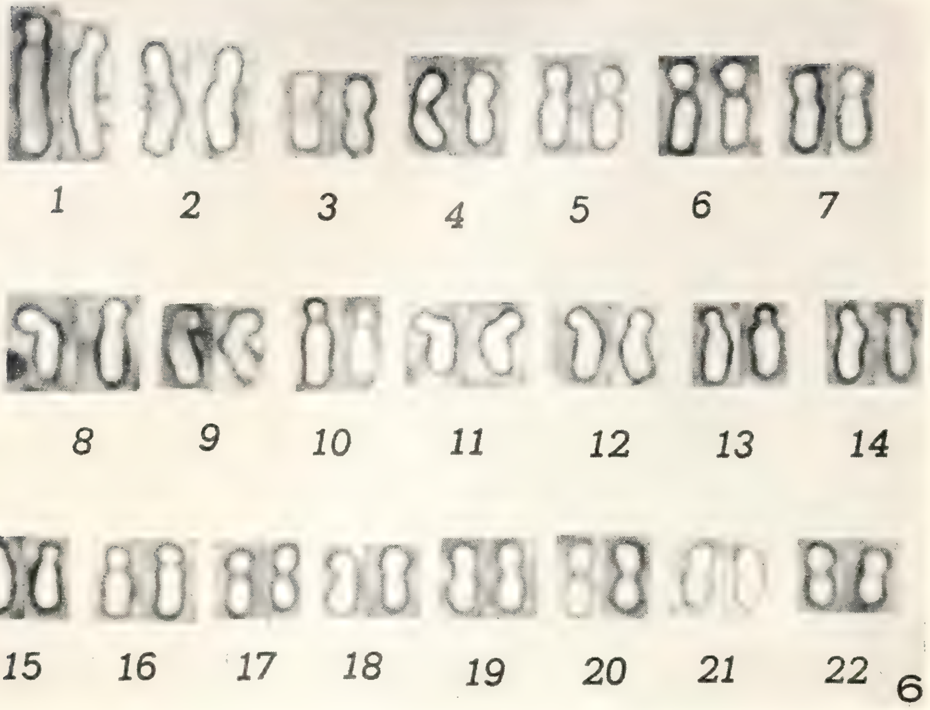


FIGURE 6. Karyotype obtained from the pre-metaphase chromosome spread preparation of Figure 2. Total magnification of the chromosomes, approximately 4000 ×.

Echinarachnius parma. The chromosomes of the egg of the sand dollar also were examined by this method. These much larger eggs are more difficult to study because of the wide area of dispersion of the chromosomes in the cytoplasm in spread preparations. Counts gave either 44 or 46 chromosomes for the diploid number. The chromosomes of the sand dollar are somewhat larger than those of the sea urchin, and the centromere position tends to be more medial. An unequivocal karyotype still cannot be determined with any accuracy.

TABLE I

Karyotype of egg of Arbacia punctulata during the first cleavage division

Chromosome pair	Description
1	Large chromosomes, nearly terminal centromeres
2	Chromosomes not as large, more medial centromeres
3–4	Medium size chromosomes, approximately median centromeres
5–9	Medium size chromosomes, sub-median centromeres
10–14	Medium size chromosomes, nearly terminal centromeres
15–16	Smaller chromosomes, nearly terminal centromeres
17–19	Short chromosomes, sub-median centromeres
20	Short chromosomes, more medial centromeres
21–22	Very short chromosomes, approximately median centromeres

Discussion

A number of widely differing values have been estimated as the chromosome number of *Arbacia punctulata*. These vary from as low as 32 (Harvey, 1940) to as high as 40 (Tennent, 1912). In a discussion of these results, Harvey (1956, p. 102) concludes that most sea urchins, including *A. punctulata,* have 36 to 38 chromosomes, in agreement with the number reported by Matsui (1924). Baltzer (1910), however, found 40 chromosomes in a related species, *A. lixula*. It is important to realize that in all these early studies the chromosome counts were made on sectioned material, which introduces many difficulties and uncertainties.

More recent studies indicate a higher chromosome number, namely 42 to 44. Nishikawa (1961), on the basis of examining the meiotic divisions of spermatogonia, found a haploid number of 21, or a diploid value of 42, for two species of Japanese sea urchins, *Hemicentrotus pulcherrimus* and *Anthocidaris crassispina*. The number, 44, reported in the present study, has also been confirmed recently by German (1964), who examined the chromosomes of *A. punctulata* in the blastula stage blastomeres by the standard acetic orcein squash technique. German described attenuation at the kinetochore region of the metaphase chromosomes, so that it is likely that the lack of centromere attachment in the present work is due to the new technique and is not characteristic of sea urchin chromosomes.

It may be that most echinoderms have a similar chromosome number and do not vary as widely as shown in the summary compiled by Makino (1951). One reason for this assumption is the chromosome number of *E. parma,* found in the present work to be 44 or 46, which is more in line with that found in *Arbacia,* and varies considerably from the earlier value of 52 determined by Matsui (1924).

The method here reported should prove useful to workers dealing with physical studies on marine eggs, especially *Arbacia,* during the early cleavage divisions. It makes it possible to determine, without sectioning the eggs, the degree of synchrony and the exact stage of mitosis (Fig. 1) at the time of an experimental treatment.

Summary

A method has been developed to stain the chromosomes and nuclei of unsectioned sea urchin and sand dollar eggs during the early cleavage divisions. The chromosome number of *Arbacia punctulata* is 44, and a karyotype of the pre-metaphase chromosomes of the first cleavage division has been made. The chromosome number of *Echinarachnius parma* is 44–46 and no karyotype has as yet been made.

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PHOTOPERIODIC CONTROL OF A PHYSIOLOGICAL RHYTHM[1]

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A hormone, proctodone, was recently discovered to be produced by epithelial cells located in the anterior portion of the proctodeum of the larval European corn borer, *Ostrinia nubilalis* (Hübner) (Beck and Alexander, 1964a). Proctodone appeared to be responsible for the activation of the neurosecretory processes leading to the production of the prothoracotropic hormone, thereby constituting a major endocrine component of the physiological processes underlying diapause development and prepupal morphogenesis (Beck and Alexander, 1964b). Cytological evidence of daily secretory cycles in the proctodone-producing epithelial cells was obtained, and some of the possible relationships between rhythmic physiological functions and extrinsic photoperiodic signals have been discussed (Beck, 1964).

In the earlier publications, cited above, it was postulated that the rate of diapause development may be determined by the degree of synchronization between two or more interacting or interdependent physiological rhythms, such that when the rhythms were physiologically "in phase," development was rapid and diapause was soon terminated. Conversely, diapause development was slow and the diapause state greatly prolonged when the participating rhythmic functions were physiologically "out of phase." In support of this "phase theory" of developmental control, evidence was presented of the existence of secretory rhythms in both proctodone-producing epithelium of the proctodeum and lateral neurosecretory cells of the larval brain (Beck, 1964). The rhythms were found to be influenced by the environmental photoperiod, with the neurosecretory rhythm being sensitive to the onset of light and the proctodeal rhythm being sensitive to the onset of darkness. Both rhythms were found to be characterized by the possession of an 8-hour period, rather than the expected 24-hour period. Since the rhythms ran through three full cycles per day, they cannot be classified as circadian rhythms, although they certainly utilized circadian photoperiodic signals as "Zeitgeberen."

The present study was undertaken in an effort to clarify some of the relationships between photoperiodic signals and the temporal characteristics of the proctodeal secretory rhythm. Emphasis has been on the cytological changes involved in the rhythmic function, rather than on the role or fate of the physiologically active substances produced during the secretory process. Although a number of physiological processes, including some endocrine functions, have been shown to display rhythmicity in plants, invertebrates, and vertebrates, there has been a marked paucity of cytological evidence in support of the hypothesis that secretory processes may show rhythmic characteristics (Halberg, 1960; Harker, 1960; Bünning, 1963; Beck, 1963; Wolfson, 1964).

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METHODS AND MATERIALS

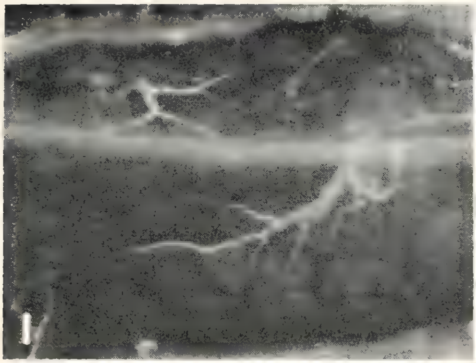
The experimental insects employed in this investigation were larvae of the European corn borer. The larvae were reared on meridic diets as previously described (Beck, 1962), with diapause being induced by means of short-day photoperiods (12 hours of light and 12 hours of dark) during the larval growth period. Some experiments involved exposure of larvae to long-day conditions, which was accomplished by means of incubators programmed for 16 hours of light and 8 hours of dark per day. All of the short-day and long-day incubators were synchronized so that the clock times of the onset of light were identical. The photoperiodic conditions differed, therefore, only in the clock times at which the lights-off signal occurred. With only a few exceptions, noted specifically below, the temperature used for rearing, treatment, and post-treatment was 30° C.

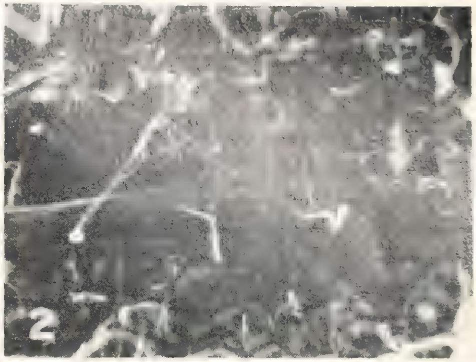
Tissue samples for permanent slide preparation were fixed in Bouin's fixative, imbedded in paraffin, sectioned at 7 microns, and stained. Staining was by either iron hematoxylin and eosin or the paraldehyde-fuchsin technique of Cameron and Steele (1959). Nearly all of the experiments involved the examination of fresh proctodeal tissue for the presence of intracellular secretory granules. This was accomplished by dark-field fluorescence microscopy; a Leitz ultraviolet accessory was used, employing a UG-1 2-mm. exciter filter and Euphos barrier filters. Living larval proctodeums were dissected out, and excess fat and tracheae removed. They were then split laterally, mounted flat in water on a microscope slide, and covered with a thin coverslip.

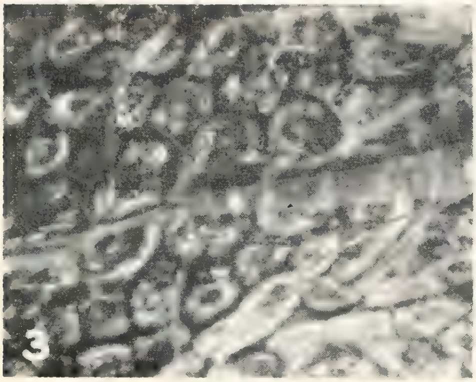
The secretory granules occurring in the proctodeal epithelium were previously observed to display autofluorescence, leading to the detection of cyclic cell activity (Beck, 1964). In the present study, the secretory cycle was followed by the examination of tissue samples collected at different times during the photoperiodic cycle. Permanent records of the relative intensity of the fluorescence and the cellular disposition of the fluorescing granules were obtained by photomicrography. For this purpose, 35 mm. color film was used (High-Speed Ektachrome, ASA = 160) with a standard shutter speed of 120 seconds. The pictures were developed and mounted as 2 × 2 inch transparencies. All subsequent considerations of the secretory state of the samples were on the basis of the colored photomicrographs, as the tissue samples themselves could not be preserved.

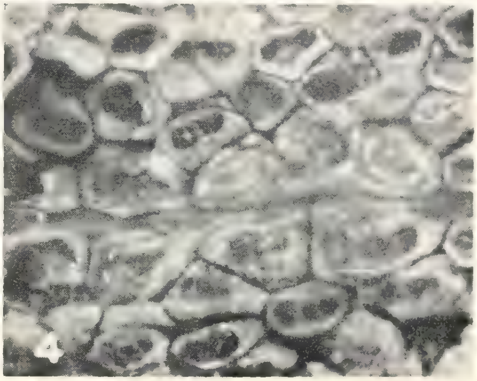
In order to study the secretory cycle and the effects of variables on the cycle, it was necessary to devise a method for comparing the secretory state of different tissue samples. For this purpose, the secretory cycle was divided into a sequence of 5 arbitrary stages based on the characteristics of the fluorescence recorded in the colored photomicrographs. Black and white prints of the photomicrographs constituting the standard examples of each stage are presented as Figures 1–5. The general distinguishing characteristics of each of the several stages are given with the figures. Although subject to the limitations invariably accompanying an atomistic consideration of a continuum, the use of this arbitrary scale of relative secretory activity has facilitated the study and has yielded consistent and reproducible results.

With only few exceptions, all of the epithelial cells of a given proctodeal sample were virtually identical in respect to the observable fluorescent inclusions, making









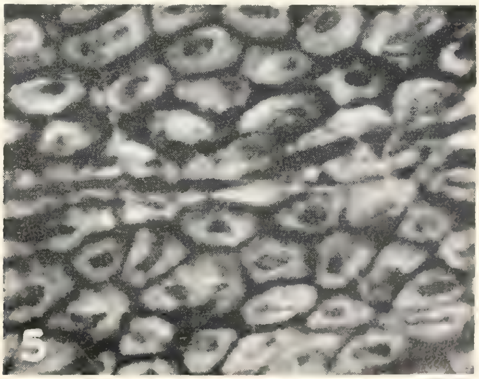


FIGURE 1. Stage 0 of proctodeal secretory cycle. Cells are devoid of visible fluorescent cytoplasmic granules.

FIGURE 2. Stage 1 of proctodeal secretory cycle. Cells contain scattered fluorescent granules; cell outlines are just discernible.

FIGURE 3. Stage 2 of proctodeal secretory cycle. Cells contain numerous large fluorescent granules; cell and nuclear outlines easily discernible.

FIGURE 4. Stage 3 of proctodeal secretory cycle. Fluorescent cytoplasmic granules vary numerous; nuclear outlines well-defined.

FIGURE 5. Stage 4 of proctodeal secretory cycle. Cells packed with finely divided fluorescent inclusions; nuclei partially obscured by inclusions.

it relatively easy to assign each preparation to one or another of the stage classifications. The procedure for assigning secretory stage values to the photomicrographs was as objective as practicable. The pictures obtained in an experiment or series of tissue samples were marked with code numbers, randomized, and compared individually with the series of standards. On the basis of such comparison, each was assigned to one of the arbitrary stages. Following this evaluation, the photomicrographs were decoded, and the stage values were recorded according to the experimental series to which they belonged. Statistical analyses of the data so obtained were limited to frequency distribution considerations. The use of statistical tests, such as analyses of variance, was avoided because of the mathematical artificiality of numerical data describing arbitrary classifications.

Results and Discussion

Rhythm characteristics

Stained sections of the anterior portions of the proctodeums of mature diapausing European corn borer larvae usually disclosed that the epithelial cells contained granular cytoplasmic inclusions that stained dark purple with paraldehyde-fuchsin. The presence of such granules, irregular multilobate nuclei, and numerous small cytoplasmic vacuoles was interpreted as being indicative of much synthetic activity. In some specimens, the cells were devoid of the stainable granules, although showing the typical vacuoles and multilobate nuclei. Whether or not the epithelial cells contained stainable secretory granules appeared to depend upon the time of day that the larvae had been fixed. These observations suggested that secretory activity was on a cyclic basis. Our interpretation has been that the paraldehyde-positive products accumulate in the small cytoplasmic vacuoles, and are then secreted into the hemolymph through the basal cytoplasmic membrane and the basement membrane underlying the cell layer. In the immature, feeding stages of the insect, these cells are known to absorb water and digested substances from the gut lumen and to secrete them into the hemolymph (Wigglesworth, 1950). During diapause and postdiapause, the larvae do not feed and the gut tract is quite empty. Our observations indicate, however, that the cells retain the capability of secretion into the hemolymph, and have an endocrine function.

Study of the secretory cycle has been greatly facilitated by the finding that the secretory granules display a pale green autofluorescence in the fresh state. The cycle of elaboration and secretion could be followed by the examination of successive timed tissue samples without the laborious delay involved in fixation, embedding, sectioning, and staining. It was found that at the stage of the secretory cycle in which no paraldehyde-fuchsin-staining granules were present, no fluorescent granules were detectable. This observation led us to the tentative interpretation that the stainable particles and fluorescent particles were identical.

The absence of fluorescent inclusions in the epithelial cells was characterized as Stage 0 of the secretory cycle (Fig. 1), and was interpreted as being the result of the cells' having liberated their accumulated secretory products. For reasons to be discussed below, this stage of the secretory cycle is thought to be of relatively short duration. After Stage 0, the cells again begin to accumulate secretory products, as shown by progressive increase in the amount of fluorescent granules—

Stages 1, 2, 3, and 4 (Figs. 2–5). Our observations have led us to believe that the cells are capable of liberating their products after they reach either Stage 3 or 4. For the purposes of studying the effects of photoperiod on the secretory cycle, Stages 0 and 1 were of the greatest interest, and their occurrence was contrasted to the occurrence of the other secretory stages.

Proctodeal tissue samples were taken hourly from groups of diapausing borer larvae that were maintained under either long-day or short-day photoperiods. The results of typical 24-hour series are shown in Figures 6 and 7, in which each tissue sample examined is symbolized by a small circle, with the occurrence of Stages 0 and 1 being depicted by solid black circles and the other stages by open circles.

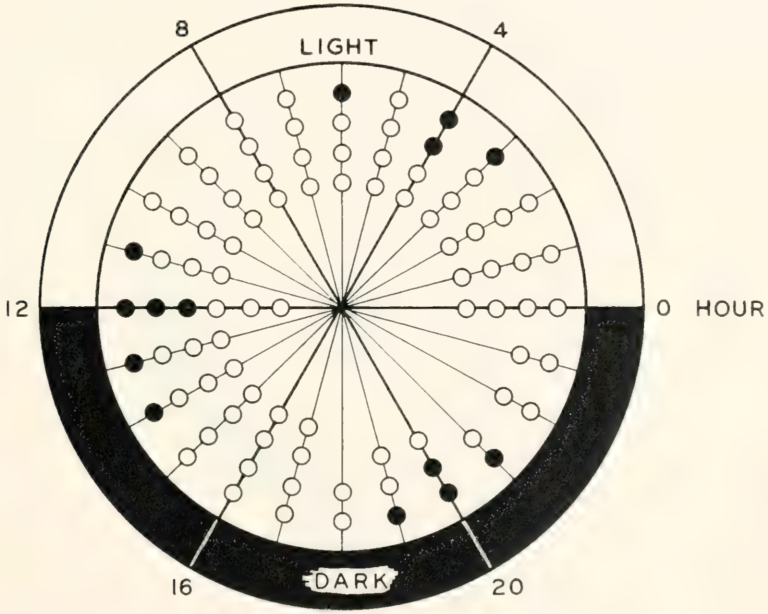


FIGURE 6. Temporal distribution of proctodeal secretory cycle Stages 0 and 1 (solid circles) among diapausing larvae of the European corn borer held in a short-day photoperiod.

It was apparent that Stages 0 and 1 did not occur at random during the 24 hours, but tended to be most frequent at the beginning of the scotophase (dark phase of the photoperiod) and at 8-hour intervals thereafter. The borer larvae used in the series depicted in Figure 7 had been transferred from a short-day photoperiod to a long-day photoperiod 10 days prior to the experiment. The results indicate, therefore, that the insects were capable of adjusting the secretory cycle in accord with the photoperiod and that the beginning of the scotophase determines the time of occurrence of the subsequent secretion times. We have confirmed this conclusion by the results of a number of experiments in which the borer larvae were required to adjust to changes in the time of the beginning of the scotophase; the secretory rhythm always shifted so that one of the times of secretion (Stages 0 and 1) approximately coincided with the onset of the scotophase. The proctodeal secretory rhythm is temporally adjusted through the insect's response to the lights-off stimulus provided by the extrinsic photoperiod.

The proctodeal secretory rhythm is not, in itself, a circadian rhythm, because it displays a period approximating 8 rather than 24 hours. It is phase-set (temporally adjusted) by the 24-hour rhythm of daylight and darkness, and is most certainly a component of the insect's photoperiodism. The possible significance of the effects of lights-on and lights-off stimuli on 8-hour physiological rhythms in the control of growth processes was discussed in an earlier paper (Beck, 1964).

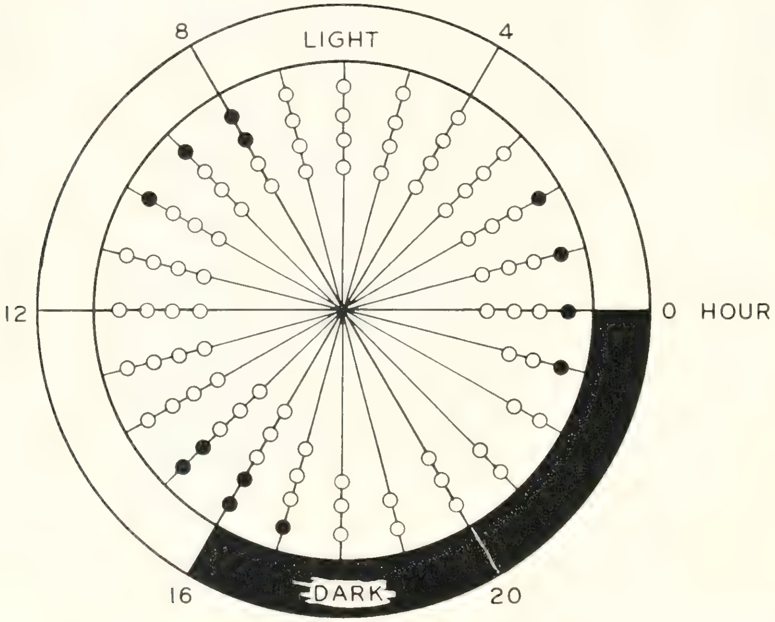


FIGURE 7. Temporal distribution of proctodeal secretory cycle Stages 0 and 1 (solid circles) among diapausing larvae of the European corn borer held in a long-day photoperiod.

Data such as those of Figures 6 and 7 gave rise to some serious questions concerning the actual time-course of the physiological rhythm. One such question pertained to an explanation of the relatively low frequency of occurrence of Stages 0 and 1 at the beginning of the scotophase. Not all (or even most) of the larvae sacrificed at that time showed proctodeal tissue that was nearly devoid of fluorescent granules. One might expect that the incidence of Stages 0 and 1 would be much higher than was actually observed, if the population was to be considered reasonably homogeneous and if all of the larvae were responsive to the photoperiodic stimuli. These considerations prompted us to undertake a more detailed study of the distribution of secretory stages during the hours immediately before and after the beginning of the scotophase.

The percentage incidence of the several arbitrary stages of secretion for some different time periods is shown in Figure 8. "Midcycle" is the 60-minute period between 4.5 and 3.5 hours prior to the lights-off time; "−1 hr." identifies samples examined between 90 and 30 minutes prior to the lights-off time; "0 hr." designates samples taken from 30 minutes before to 30 minutes after the lights-off time; and "+1 hr." designates tissue samples collected from 30 to 90 minutes after the beginning of the scotophase. About 40 larvae were dissected and examined to obtain the data for each of the different time periods.

The "midcycle" distribution shows Stage 3 to be the most frequent, with Stages 3 and 4 constituting the condition of over 80% of the insects examined. At this time there were no Stage 0, and Stage 1 was rare (one instance out of a total sample of 40 larvae). At "−1 hr." the distribution changed only in that Stage 0 and Stage 1 were each present to the extent of about 5%. The distribution at "0 hour" showed two pronounced changes: (a) the incidence of Stage 1 was greatly increased, and (b) the incidence of Stage 3 was much lower than in previous samplings. The distribution at "+1 hour" showed a decline in the incidence of Stage 1 and an increase in Stage 3. Although not included in Figure 8, histograms of the incidence of the different secretory stages at +2 and +3 hours showed a return to the "midcycle" distribution.

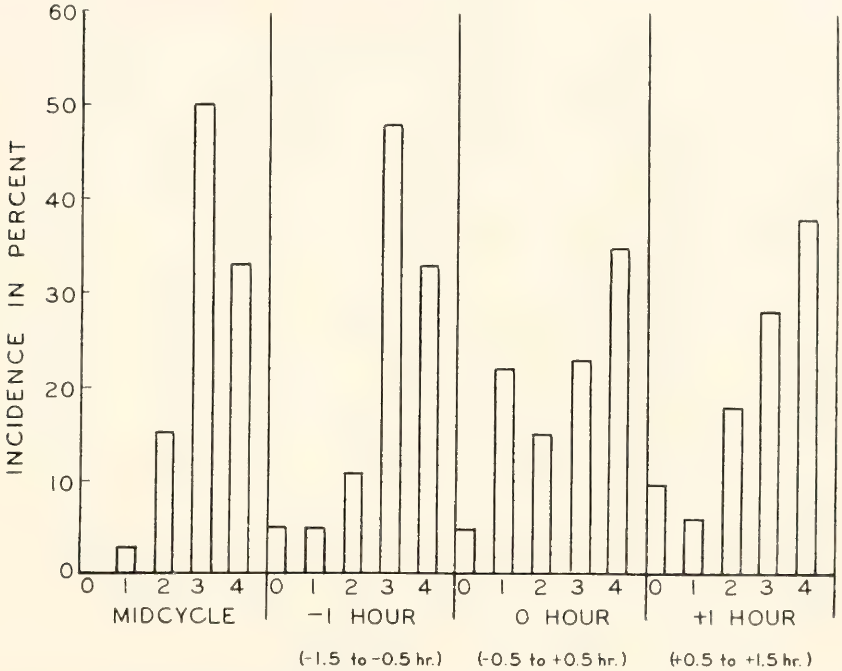


FIGURE 8. Incidence of different arbitrary proctodeal secretory stages (0–4) among European corn borer larvae examined at different times during the secretory cycle.

The cell state observed in Stage 0 and Stage 1 proctodeal tissue samples was interpreted as an indication that the cells had liberated their secretory products shortly before the larvae had been dissected. For this reason, the incidences of these two secretory stages were considered together, and their incidence compared to the incidence of the other arbitrary stages in subsequent experiments. Combining the data from a number of series in which the larvae examined were from the standard short-day photoperiod, the temporal distribution characteristics of Stages 0 and 1 were determined for the period from two hours before to two hours after the beginning of the scotophase (Fig. 9). Of the total number of Stages 0 and 1 observed, 43% occurred within 30 minutes of the beginning of darkness. The data form an obviously normal frequency distribution, with the mean falling very close to the lights-off time of the photoperiodic cycle. From these data, we can conclude that the insect's proctodeal secretory rhythm is, indeed, temporally adjusted to the beginning of the scotophase, such that the proctodeal cells tend to release their secretory products in approximate synchrony with the lights-off signal. Because the secretory rhythm appears to possess an eight-hour period, it is evident that photoperiod can directly influence only one of the three daily cycles; the others must occur endogenously, but timed in accord with the cycle that responded to the photoperiod.

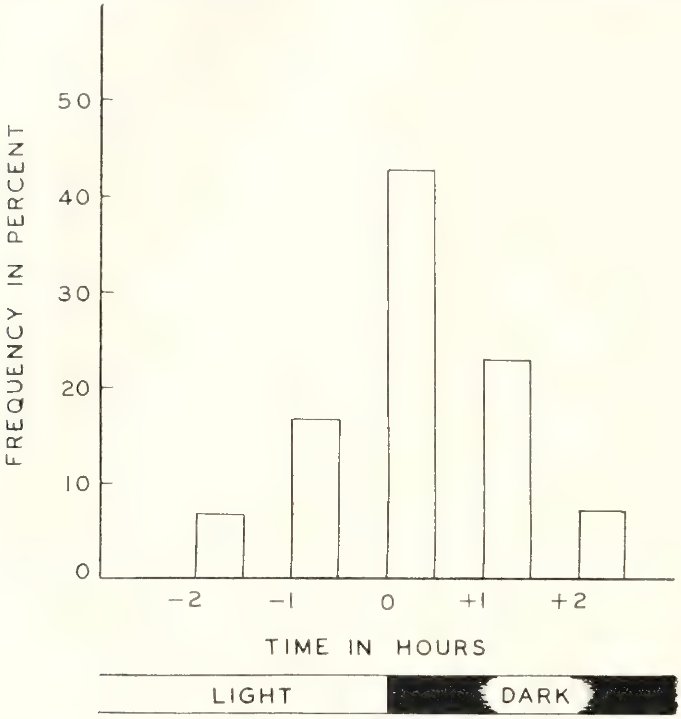


FIGURE 9. Frequency distribution of combined Stages 0 and 1 during the hours surrounding the beginning of the scotophase.

Sampling probability characteristics

If the proctodeal cells liberate their secretory products in approximate synchrony with the lights-off signal, why is the incidence of Stages 0 and 1 often relatively low, even at the "0 hour" as shown in Figure 8? This effect can be explained on the basis of the unfavorable probabilities attending the tissue sampling procedure. The measured response (Stages 0 and 1) is assumed to form a normal distribution such that two-thirds of the larvae may be expected to respond within 60 minutes on either side of the lights-off time. A second assumption is also made: the duration of Stage 0 is about 15 minutes and that of Stage 1 is about 30 minutes. When a larva is chosen at random for dissection at about the beginning of the scotophase, the probability that it will be among those that respond within the two-hour period designated above is 0.67. The probability that it will be at Stage 0 at the time of dissection is 0.67 × 0.125 (because there are eight 15-minute periods in two hours). This means that the probability of observing a Stage 0 tissue preparation at even so favorable a time is but 0.083; on the average, one larva out of a sample of twelve should be found to be at Stage 0. If the duration of Stage 1 is twice that of Stage 0, about one larva out of six should be found to be at this stage when the population is sampled during the hour before and the hour after the lights-off time. The expected incidence of Stages 0 and 1 combined would be about 25% of the larvae examined; the "0 hour" distribution data in Figure 8 show a combined Stages 0 and 1 incidence of 28%.

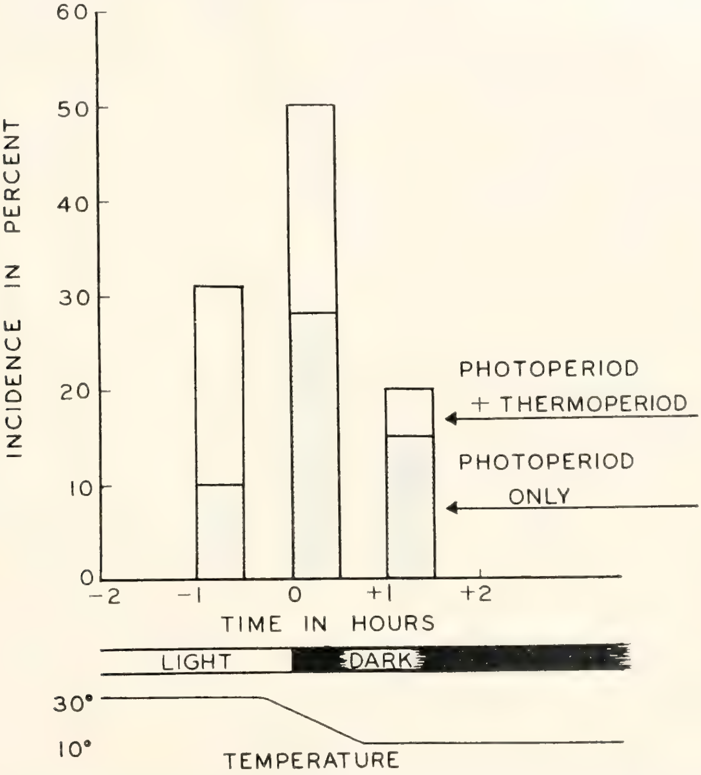


FIGURE 10. Effect of a combined thermoperiod and photoperiod on the incidence of Stages 0 and 1 of the proctodeal secretory cycle in diapausing larvae of the European corn borer.

These probability considerations have some important implications that reach beyond the present limited study. In any instance where a physiological process cannot be followed continuously in each individual specimen, the investigator is limited to making observations on a succession of specimens, each of which has been fixed at some point of time. Under such conditions, especially those in which successive physiological stages are relatively fleeting, the existence of highly significant rhythmic functions may be overlooked or, if suspected, difficult to demonstrate convincingly. In the present investigation, the demonstration of a photoperiodically sensitive secretory rhythm could not have been accomplished in the absence of the moderately large numbers of samples made feasible by the simple autofluorescence technique of examination.

Effects of thermoperiod

An attempt was made to increase the incidence of Stages 0 and 1 among the borer larvae at the time of the lights-off signal. A thermoperiod was superimposed on the photoperiod, so that the incubator temperature fell from 30° C. during the photophase to 10° C. during the scotophase. Previous results (Beck, 1962) had shown that low temperatures during the scotophase increased the incidence of diapause, whereas high temperatures at that time tended to prevent diapause. As shown in Figure 10, combining a thermoperiod with the short-day photoperiod had the expected result of greatly increasing the incidence of observed Stages 0 and 1. The large increase in response that occurred at one hour before the light-off signal indicated that the effect was not a simple prolongation of the duration of Stages 0 and 1 because of low ambient temperature. It seems more likely that the effect of the thermoperiod was one of decreasing the time range of the response, and thereby improving the probability of observing specimens in Stages 0 and 1 during the two-hour period of interest.

Short-day to long-day transition

That the secretory rhythm was capable of adjusting to a changed photoperiodic schedule within a 10-day period was apparent early in the study (Figure 6 compared to Figure 7). Attempts to trace the detailed course of such a transition have not been wholly successful, however, because of the limitations of our sampling methods and the inherent variability among the individuals of the experimental populations used.

A large group of borer larvae that had been reared under a short-day schedule in which the lights-off signal came at 12 noon was transferred to a long-day schedule in which the lights-off signal came at 4 PM. The incidence of Stages 0 and 1 was determined for three time periods: (a) 11 AM to 1 PM, which was one hour on each side of the lights-off time of the original short-day photoperiod; (b) 1:30 to 2:30 PM, which was midway between the old and new scheduled lights-off time; and (c) 3 to 5 PM, which was one hour on each side of the lights-off signal of the newly imposed photoperiod. The data of Figure 11 are typical of the results obtained from experiments of this type. The incidence of larvae responding in accord with the original noontime lights-off signal declined during the 8 days of the experiment. Responses during the 1:30 to 2:30 interval were observed throughout the 8 days, with the incidence about constant from the second day on. As expected, no larvae responded between 3 and 5 PM on day 0, but the incidence of response increased from day 1 until at least day 6. The results indicate that there was considerable individual variation in the larvae's ability to adjust to a four-hour change in the photoperiod. Some larvae were responsive to the new photoperiod by the second day, whereas others were still in the process of making the transition on the 8th day, as evidenced by the incidence of responses occurring in the middle hour of 1:30 to 2:30 PM. Although it was apparent that transient phases occurred during the process of becoming synchronized with a changed photoperiod, we were unable to determine the number of transient responses involved. Nonetheless, these results are in very good agreement with those of earlier work (McLeod and Beck, 1963), in which the effect of photoperiod on diapause development was studied. In that work, transfer of diapausing borer larvae from short-day to long-day photoperiods and from long-days into continuous darkness disclosed that some of the larvae could adopt and maintain a long-day developmental schedule within two days, but at least 10 days were required for all of the population to make the adjustment.

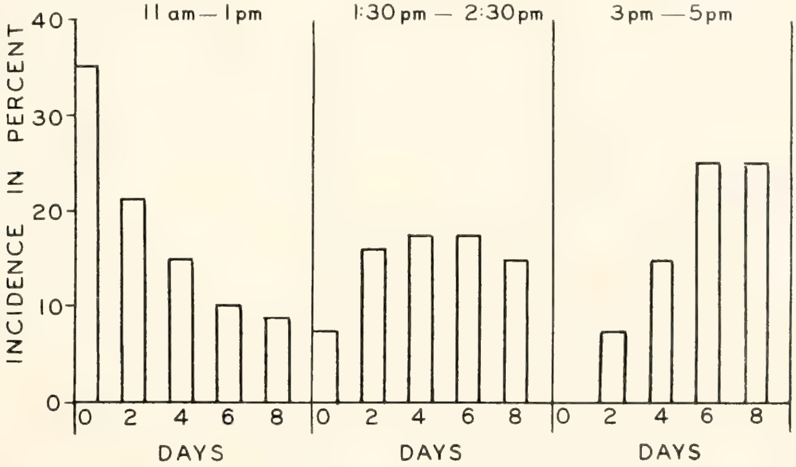


FIGURE 11. Incidence of secretory Stages 0 and 1 during 8 days following a four-hour change in the time of the lights-off signal (European corn borer larvae in diapause).

SUMMARY

1. In mature diapausing larvae of the European corn borer, *Ostrinia nubilalis* (Hübner), the epithelial cells of the anterior portion of the proctodeum were observed to show evidence of a regular cycle of synthesis and secretion. The cycle involved progressive accumulation and subsequent disappearance of paraldehyde-positive, autofluorescent cytoplasmic inclusions. For the purposes of this study, the secretory cycle was divided into a series of 5 arbitrary secretory stages.

2. The proctodeal secretory activity was shown to constitute a rhythmic function. The rhythm was found to be non-circadian, in that it displayed an approxi-

mately 8-hour period, such that three complete secretory cycles occurred during each 24-hour day.

3. The proctodeal secretory rhythm was shown to be phase-set by photoperiod. The release of secretions (loss of intracellular granules) occurred in response to the beginning of the scotophase (lights-off stimulus) and endogenously every 8 hours thereafter.

4. A thermoperiod that was superimposed on the photoperiod, so that a low temperature was concurrent with the scotophase, had the effect of intensifying the physiological response to the photoperiod.

5. From two to ten days were required for the secretory rhythm to re-synchronize with a four-hour change in the clock time of the lights-off signal. Because of great individual variability, the number of transient phase responses could not be determined.

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THE EGG COCOONS OF SCOLOPLOS ARMIGER O. F. MÜLLER

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In his review entitled "La ponte et l'incubation chez les annélides polychètes," Gravier (1923) listed 12 species which are known to lay their eggs in an agglomeration but which do not incubate them. Several more species have since been shown to spawn similarly (Herpin, 1925; Aiyar, 1931; Wilson, 1932; Day, 1934; Thorson, 1946; Bookhout and Horn, 1949; Rullier, 1954; Pillai, 1958) but no study of the chemical and physical nature or ecological role of the egg cocoons seems to have been published. The present paper is concerned chiefly with the spawning of *Scoloplos armiger* and the chemical nature of the cocoon jelly.

S. armiger has the same general distribution over the shore as *Arenicola marina* (Thamdrup, 1935; Linke, 1939). The egg cocoons are found where the worms normally live, on the gently sloping muddy sand of the foreshore which does not dry out at low tide. They have often been described, for example by Cunningham and Ramage (1888), Ehlers (1892), de Groot (1907), Thamdrup (1935), Linke (1939), Thorson (1946) and Smidt (1951). At the time of formation the cocoon is a pear-shaped or globular mass of firm jelly, about 1 to 1.5 cm. in diameter and containing from 500 to 1200 eggs, and is inserted into the sand by a stalk about 6 cm. long. Since a mature female may contain between 3000 and 5000 eggs, it looks as if a worm may form more than one cocoon (Smidt, 1951).

SPAWNING

Numerous authors, for example Mau (1882), Thorson (1946) and Anderson (1959), state that spawning occurs in the spring and lasts for about a month, its exact dates varying with the particular district. At Whitstable spawning takes place in February or March and lasts little more than a week. The method of forecasting the spawning period by the measurement of oöcytes showed, during the winter 1949–50, that spawning might be expected in February, 1950. Its onset occurred on February 18, 1950, with a suddenness which is shown by the following observations.

During a careful scrutiny of a wide area of the shore at Whitstable, lasting from 08.00 to 10.00 hr. on 18 February, only 5 (total) cocoons were seen. After the shore had been covered by the midday high tide an average of two to three cocoons *per square meter* was counted in the same locality. At 10.00 hr. on February 19 cocoon counts gave an average of 5 per sq. m. but by the evening low tide of the same day the count had risen to an average of 23 per sq. m.

In other years spawning began on the days listed and coincided with the spring tides of new (●) or full (○) moon. 1949, 15 Feb. ○; 1950, 18 Feb. ●; 1952, 29 Feb. ●; 1953, 1 Mar. ○; 1954, 20 Feb. ○; 1955, 10 Mar. ○; 1956, 14 Mar. ●.

In 1964 spawning occurred at Whitstable on 29 Feb. (Gibbs, private communication) while at Southend-on-Sea, on the opposite side of the Thames Estuary, a few worms spawned in February but the massive spawning did not occur until about 24–25 March.

Formation of the cocoon

Cocoon formation has not been achieved in the laboratory and is difficult to observe on the shore, as it occurs at high tide. It is not known how many worms take part in the formation of a cocoon but indirect evidence suggests that male and female worms come together in a burrow during spawning so that the eggs are fertilized as they leave the terminal glandular portions of the nephridia of the female. It is these organs which secrete the jelly which envelops the worms and their eggs while they are still within the burrow. On one occasion two worms were found within the stalk of a new cocoon and a search in the sand immediately below newly-formed cocoons usually revealed male and female worms occurring close together. The sex ratio, male to female, is approximately 1.3:1.

The shape of the cocoon, that of a single large drop of a viscous substance emerging from a tube, is clearly due to its method of formation. Rarely are malformed cocoons found although occasionally two smaller drops are attached to a single stalk and more rarely an additional stalk is found, as if one of the worms entered or left the cocoon by a different route. On one occasion male and female worms spawned in the laboratory soon after collection and before they had established their proper burrows. The cocoon jelly was released below the surface of the sand in irregular masses, emphasizing that the form of the cocoon normally results from its method of formation as a drop emerging from a tube.

De Groot (1909) stated that copulation occurs, but Anderson (1959) was unable to find sperm or fertilized eggs in the coelom of the female. Observations made on stained sections of fixed ripe females confirm this; neither were living spermatozoa seen in the jelly of freshly-laid cocoons although the eggs which they contained were fertilized. It looks, therefore, as if the eggs are fertilized immediately they leave the body cavity of the female before the jelly has had time to swell.

THE COCOON JELLY

Source of the material

The jelly of which the cocoon is formed is provided by the female and comes from the specialized end portions of the nephridia, of which about 100 pairs in the middle region of the body develop conspicuous white swellings at the bases of the notopodia (Eisig, 1914). These glandular portions surround the nephridiopores and consist of very large cells, each of which is greatly distended in the breeding season with aggregations (measuring 20–30 μ in diameter) of rod-like bodies, each 5–10 μ long (Fig. 1).

Formation of jelly from rodlets

When temporary preparations of the glandular nephridial cells are made and squashed or teased in sea water, some aggregations of rodlets may be forced out intact but many are broken up into their constituent rodlets. In such a preparation some of the rodlets appear to explode suddenly and at random but a rodlet can sometimes be seen to swell a little before suddenly bursting. Under phase-contrast illumination the rodlets are sufficiently dense to appear quite black but the diffuse body formed after bursting can only just be made out. No ghosts or envelopes of rodlets are left, so that they appear to be simply a concentrated form of the jelly material which is able to absorb water and greatly to increase in volume.

The effect on the rodlets of various reagents was watched, using phase-contrast illumination, during irrigation under a cover glass or in hanging drop preparations. The tests were made about two weeks before spawning took place.

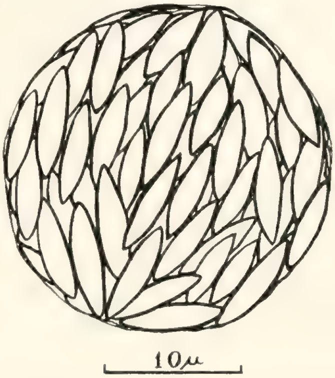

FIGURE 1. Aggregation of rodlets from glandular portion of nephridium of female.

Rodlets mounted in hanging drops of 100%, 80% and 60% S.W. were mostly intact after 5 hours but burst in that time in 40% S.W. In 20% S.W. all burst within 13 minutes and in distilled water within 3 minutes. In S.W. made alkaline to pH 11 with NaOH, in N H_2SO_4 and in S.W./N H_2SO_4:1/1, all burst quickly but in S.W./N H_2SO_4:4/1 most remained intact. In Bouin's fluid all the rodlets burst quickly but in 5% formalin in S.W. only some burst. Subsequent irrigation with distilled water caused them all to burst. Surface-active agents in sea water, both ionized (0.1% Teepol) and un-ionized (0.1% Triton × 100), caused the rodlets to burst. Teased male *Scoloplos* in S.W. had no visible effect. The conversion of the rodlets into jelly can thus be accelerated by various treatments but none of the tests throws much light on the natural process, which is rapid and once started goes to completion. Even in the tests with dilute S.W. there was never any suggestion of the rodlets taking up an intermediate size in equilibrium with a medium of osmotic pressure different from that of their normal environment.

Microscopical appearance

The cocoon jelly can be seen under the microscope (especially with phase-contrast) not to be perfectly homogeneous but somewhat fibrous in appearance. This also shows up in the fixed material stained with Heidenhain's Azan stain (blue), Mallory's stain (blue), the P.A.S. stain (red), muci-carmine (red) and

Alcian blue. Metachromasy is shown with toluidine blue. The fibrous appearance is in no way due to the presence of clearly defined fibers but rather to what are best described as local condensations of the jelly.

Chemical properties

The general chemical nature of the jelly is indicated by its staining properties. All these point to its being (or at any rate containing) a mucoprotein. Further tests were made in an attempt to find out details of its chemical composition and, if possible, to relate this to its physical properties and biological role.

Some 2000 cocoons were collected, teased up by hand, using needles, centrifuged at 5° C. for 20 minutes at 20,000 *g* and the jelly retained, the eggs and debris in the lower fraction being discarded. The process was repeated after further teasing of the jelly and a clean, egg-free jelly was obtained. Examined under the microscope it was seen to contain only scattered specks of unidentifiable material probably derived from the substratum. Any jelly that was not used fresh was stored in 50% isopropanol in a refrigerator until required.

Rough determinations of the organic content of the centrifuged native jelly were made by weighing a sample, washing it at least 8 times in distilled water until demonstrably chloride-free when tested with silver nitrate, and then drying to constant weight in air on a hot-plate. Values of the salt-free dry weight were 1.5% and 0.5% of the wet weight, the difference probably being due to the different amounts of the watery fluid which had been squeezed out by centrifugation before weighing, as well as to possible inequalities in drying. The jelly does not dissolve in sea water, distilled water or dilute acids. It dissolves in 0.5 N NaOH with gentle warming but it seems likely that this process brings about considerable degradation.

Nitrogen content

The nitrogen content of the jelly was estimated as follows. A sample was taken, washed in repeated changes of distilled water until the washings gave no trace of chloride when tested with silver nitrate solution. This was evaporated to dryness, washed again with distilled water and again evaporated to dryness. It was heated at 105° C. in an oven until of constant weight and two samples were used for total nitrogen estimation by the Kjeldahl method of Yuen and Pollard (1953). The average of two estimations gave a figure of 12.4% N (12.3% and 12.5%). This figure is below that normally accepted for protein (16%) and somewhat above that for an amino sugar such as glucosamine (7.8%). It looks, therefore, as if the material is a combination of protein and carbohydrate moieties.

Amino-acid composition

A sample of the dried material weighing 12.2 mg. was hydrolyzed by heating for 7 hours at 105° C. with 10 ml. of 6 N hydrochloric acid in a sealed tube. The solution was evaporated in a rotary film evaporator and was twice redissolved and evaporated almost to dryness. The residue was taken up in distilled water and left in an evacuated desiccator over calcium chloride and sodium hydroxide to remove the last traces of hydrochloric acid. Two-dimensional paper chromatography on Whatman No. 1, using butanol/acetic acid/water (12:3:5 by volume) and phenol/water (4:1 by weight) as solvents, showed that some 15 amino acids were present. They were mostly revealed by ninhydrin and identified by comparison with the positions taken up by known amino acids and also by the method of enhancement of each spot in turn by a known amino acid applied in addition to the hydrolysate. Runs were also made using as solvents ethanol/water/ammonia (12:1:1 by volume) and methanol/water/pyridine (20:5:1 by volume). The presence of aspartic acid, proline, hydroxyproline and alanine was confirmed by the use of isatin. The following amino acids were present: alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, hydroxyproline, leucine (or isoleucine), lysine, methionine (or valine), ornithine, proline, serine, threonine, tyrosine. A feature of interest is the presence of hydroxyproline which is usually found only in collagenous tissue proteins. These frequently have mucopolysaccharides associated with them.

Carbohydrate components

Sugars were separated on Whatman No. 54 paper, in the first place by descending chromatography, using isopropanol/water (4:1 by volume). Runs were continued for 17 hours, which allowed the solvent to drip off the paper and increased the resolution of the spots with low R_f values. The sugars were revealed by silver nitrate in acetone, followed by sodium hydroxide in ethanol. The papers were bleached in ammonia and the spots blackened by hydrogen sulphide. The Elson-Morgan reagent for amino sugars was also used. These tests clearly showed the presence of glucosamine and/or galactosamine and traces of two other sugars. Additional runs in ethyl acetate/pyridine/water (12:5:4 by volume), followed by revelation with silver nitrate, showed one of the simple sugars to be glucose. The other corresponded in position to fucose (a hexose) and xylose (a pentose) which have similar R_f values in most solvents. Since the sugar was in low concentration in the hydrolysate the negative result of the phloroglucinol test for pentoses applied to the paper could not be relied upon to prove the absence of xylose. Accordingly the hydrolysate was tested for fucose, using the sulphuric acid/thioglycolic acid test for 6-deoxyhexoses (Dische, 1947), to which group of sugars fucose belongs. A positive result was obtained. Testing the hydrolysate for pentoses by Bials' orcinol reaction and the phloroglucinol test gave a negative result. From this it was concluded that the trace of sugar present in addition to glucose and revealed as having an R_f value indicating either fucose or xylose was, in fact, fucose.

The presence of both glucosamine and galactosamine was also confirmed by the ninhydrin degradation method of Stoffyn and Jeanloz (1954), followed by identification of the pentoses, arabinose and lyxose, which are produced by the reaction. This was done by paper chromatography, using ethyl acetate/pyridine/water as solvent, followed by revelation with silver nitrate and comparison with the position of known samples. The size and intensity of the spots obtained indicated that these two amino sugars were present in about equal amounts and in greater quantity than glucose and fucose.

Tests for uronic acids were made on the hydrolysate, using naphthoresorcinol. They were negative and confirmed the absence of any spot on the paper chromatogram with an R_f corresponding to that of uronic acids. The presence of sulphate groups was tested for by adding barium chloride solution to the hydrolysate. No precipitate was obtained and the sugars are therefore not sulphated.

Attempts were made to separate any possible constituents of the jelly by electrophoresis on paper and cellulose acetate membrane at pH 8.5 and pH 4.0 and 6 volts per cm., followed by detection with Schiff's reduced fuchsin sugar reagent and naphthalene black for proteins. All the tests failed to demonstrate any mobile component whether they were made on small blobs of dialyzed native jelly or on washings of the jelly concentrated by ultrafiltration through Visking tubing. All that can be concluded from this is that the molecular weight is very high or that the molecules have little net charge, but the results fit in with the other properties of the material, namely its insolubility and its extremely high viscosity at low concentration, which is suggestive of a cross-linked molecular structure.

An estimate by the anthrone method (Bangle and Alford, 1954) of the total quantity of simple sugars present in the organic component of the jelly was made, using a weighed, salt-free, dry sample. (This method gives no color with amino sugars in the concentrations present.) The intensity of color obtained was compared with that of standards from known concentrations of glucose in an EEL Spectra spectrophotometer at 625 mμ. The simple hexose component amounted to approximately 7% of the dry weight of the jelly.

From all these analytical procedures there is no doubt that the jelly can be called a mucoprotein having the carbohydrate moiety composed of galactosamine and glucosamine, glucose and fucose. One of the chief properties of such materials is their ability to bind large amounts of water to a very small amount of organic matter. The jelly of *Scoloplos* is no exception to this and was easily shown to be capable of imbibing water when dry and regaining its original volume or thereabouts. This process of drying and imbibition was repeated several times and was not affected by storage of the jelly in 50% isopropanol.

Physical properties

The bulk of the mucoprotein jelly does not dissolve in water but remains as a "blob" even after teasing and centrifugation. Moreover it cannot readily be dissolved by chemical methods which do not degrade it considerably. These properties are obviously of great importance in its role of egg container.

On the other hand its structure can be altered by certain purely physical means sufficiently to precipitate it or at least a component of it. When treated in a ground-glass testtube homogenizer, in which the jelly is subjected to considerable shearing forces, it yields a white precipitate which on drying becomes powdery and is unable to imbibe water and regain the original volume of the jelly. The liquid left after spinning off the precipitate was tested with the anthrone reagent and with the Elson-Morgan reagent for amino sugars. The anthrone test was positive but the Elson-Morgan gave only a very faint coloration. The anthrone method (Bangle and Alford, 1954) was then used to estimate the amount of hexose liberated from a sample of jelly by homogenization. By treatment in the homogenizer for various lengths of time up to 15 minutes, it was possible to split off about 5% of the dry weight of the jelly, corresponding to about 70% of the simple carbohydrate present.

From these tests it can be concluded that the gel-forming property of the material depends upon the presence of the carbohydrate component of the complex, and that this is only loosely attached and can be removed by physical means.

The tensile strength of the stalk is such that, when rapidly loaded in sea water at room temperature, it extends and breaks within seconds when a weight of about 8 g. is applied. When loaded with 1 g. or even 0.5 g. it stretches quickly at first and then more slowly but continues to extend slightly for 18 hours. Very slow extension was continuing when the experiment was stopped. When the tension was released there was an immediate shortening although not to the original length. These measurements were made using a smoked drum and light aluminum lever, and while it is clear that the jelly can be described in general terms as a viscous-elastic system, recording methods with less backlash and of greater sensitivity are needed to say how its properties may best be represented in a conventional way. In nature the cocoons may be expected to be under a slight stress during most of their life because, although the direction of the tide changes at ebb and flow, the cocoon stalk is flexible and so always takes up the same position with respect to the current. This stress is presumably less than the 0.5 g. which was shown to cause extension, but the sudden stresses to which the cocoons may be subjected by wave action would be expected to be much greater. For overcoming the effects of these the elastic element in the behavior would be effective since the forces act for only a short time.

A sample of the jelly was prepared for x-ray analysis by drying a film of the material on a glass plate. This film was peeled off and cut into rectangles 2 × 5 mm. which were stuck together by slight moistening to form a block 2 × 2 × 5 mm. The x-ray pattern perpendicular to the plane of the dried films of jelly showed disorientated protein but the x-ray pattern parallel to the dried films showed an indication of structure at the molecular level, composed of sheets separated by about 10 Å. It is tempting to suppose that these correspond to proteins which are cross-linked by the carbohydrates but further experiments are required before this can be established.

The Function of the Cocoons

The cocoon-forming habit occurs sporadically in the order Polychaeta and has thus presumably evolved many times. It is clearly based on the secretion of some part of the glandular epidermis which most annelids possess. Few authors have recorded the exact source of the cocoon jelly but the stage of hatching of the developing eggs is generally noted in the literature. It varies from the free-living trochophore, as in *Phyllodoce maculata,* or the metatrochophore, as in *Marphysa* sp., to chaetigerous larvae in *Aricia* spp. and *Scoloplos* spp.

There is no positive evidence that the laying of eggs in cocoons confers any nutritive, thermal or osmotic advantage, although the opinion has been expressed that because algae and Infusoria are frequently seen to flourish in the cocoon they provide a food supply for the larvae. Bookhout and Horn (1949) stated that the embryos of *Axiothella mucosa* fed on the diatom *Nitzschia* which multiplied in the cocoons, and Pillai (1958) is of the opinion that the embryos of *Marphysa borradailei* are nourished either by the jelly substance or the protozoans that flourish in it. It is possible that the larvae gain some nutriment from the jelly but it should be remembered that the eggs are generally packed with reserve food materials while the jelly contains only about 1% of organic material, and in *Scoloplos* and *Phyllodoce,* at any rate, the cocoons seem to be little altered when the larvae have left. Also, no evidence has been put forward to prove that the concentration of food organisms is greater in the jelly than in the substratum. In the locality in which *Scoloplos* and *Phyllodoce* occur at Southend-on-Sea, the surface of the sand is frequently much richer in diatoms than the interior of the cocoon jelly. The chief function of the cocoon appears to be to keep the young in their proper station but even this advantage is partly lost if trochophores are liberated and especially if they are positively phototactic like those of *Phyllodoce*. Further ecological studies are required to determine more precisely the role of the cocoons in the biology of their producers and what advantages, if any, the cocoon-laying habit confers.

I am grateful to Professor G. E. Newell, with whom many of the observations at Whitstable were made, to Dr. J. E. Forrest for information on the structure of the nephridia, and to Mr. A. G. Taylor who did much of the chemical analysis.

SUMMARY

1. The egg cocoons of *Scoloplos armiger* are laid at Whitstable during the few days of high spring tides in February or March.

2. The cocoon jelly is derived from secretion of cells forming the end portions of the nephridia of the female and contains only about 1% of organic matter which is mucoprotein. Fifteen amino acids, including hydroxyproline, were identified in the protein moiety and glucosamine, galactosamine, glucose and fucose in the carbohydrate portion.

3. The jelly can be dried but will swell again in water. This property can be destroyed by homogenization when 70% of the hexoses are split off.

4. It is unlikely that the cocoons play any significant part in the nutrition of the larvae, their chief function probably being to ensure that the larvae remain near a suitable substratum.

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HEART RATE AND LEUCOCYTE CIRCULATION IN CRASSOSTREA VIRGINICA (GMELIN)[1, 2, 3]

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It was noticed that leucocytes[4] in the circumpallial artery of the oyster at low temperatures tend to form aggregates which gradually become dispersed with elevation of ambient temperatures. On the basis of this observation, a hypothesis was formulated to interpret the fluctuation in the numbers of circulating leucocytes with changes in the ambient temperature in normal oysters. Assuming the number of circulating leucocytes is more or less constant in a normal oyster, to maintain the leucocytes in suspension, a certain amount of agitation must be present in the circulatory system or leucocytes will eventually settle out. Thus, the number of leucocytes in suspension is related to the intensity of agitation. The following series of experiments was designed to test the validity of the above hypothesis.

In studies of oyster experimental pathology, sampling of heart blood after intracardial injections of particulate or soluble materials is one of the established procedures. To understand the effect of this experimental manipulation on the heart rate and number of leucocytes in the blood is, therefore, of prime importance.

MATERIALS AND METHODS

1. *Oysters, aquaria, and sea water*

The oysters used in this study were 8–15 cm. in length and were collected from the Navesink River near Red Bank, New Jersey. The shells were heavily infested with *Polydora* but, aside from weakening the structure of the shell, this appeared to have no effect on the functioning of the oyster. About 30–33% of the oysters collected from this area in the summer were parasitized by *Bucephalus cuculus.*

Groups of 4–8 oysters, depending on their sizes, were placed in each aquarium (Pyrex battery jar 25 × 25 cm.) with approximately 4 liters of sea water. Water temperatures in the aquaria for experiments performed in the winter usually approximated the ambient temperatures of the laboratory, *i.e.,* 15°–21° C., by placing the aquaria in a water bath, or were held at specific temperatures by using thermostatically controlled glass immersion heaters (±2° C.) in a constant temperature room.

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[4] In the oyster the terms *amebocyte*, *leucocyte*, and *phagocyte* are used interchangeably, as suggested by Stauber (1950).

The water used to hold oysters in the laboratory was collected from the Shrewsbury River at Atlantic Highlands, New Jersey, where the salinity varied from 20 to 27‰. Since ambient salinity change (Feng, 1960) has also been shown to influence the heart rate, all experiments listed below were conducted in the sea water of 20‰ which was obtained by diluting the stock water with appropriate amounts of aged tap water. The diluted sea water was stored in a constant temperature room (5° C.) before being used. Water was changed at least twice daily and vigorous aeration in each aquarium was maintained constantly.

2. *Preparation of oysters for injection, bleeding and heart rate studies*

Oysters were prepared for intracardial injection and bleeding by filing a window directly over the heart on the left valve of the shell with a power drill equipped with a circular grinding wheel. After a small hole was made in the nacreous layer, the opening was enlarged by subsequent fragmentation of the shell. For heart rate studies, however, the left valve anterior to the adductor muscles was completely removed, exposing the entire visceral mass, since according to Stauber (1940) valve closure also influences the heart rate. Oysters thus prepared were kept at 5° C. overnight to allow recovery from the shock of the treatment. Prior to the injection, the general physiological condition of each oyster was determined by noting (1) the strength of heart beat, (2) the response of adductor muscle to a mechanical stimulus such as a gentle tap on the aquarium wall, (3) the presence or absence of water pumping and (4) the ejection of fecal strings. Conditions (2) and (4) are not applicable to partially denuded oysters for heart rate studies, since their hinges are broken and consequently they are unable to snap their shells shut. Animals which did not meet the above standards and those which were infected with *Bucephalus* were discarded.

3. *Preparation of inocula*

Seitz-filtered sea water (20‰) was used as one of the inocula.

One pound of fresh spinach was ground in a solution of 0.40 *M* sucrose, 0.05 *M* Tris, pH 7.8, and 0.01 *M* NaCl for 30 minutes at 5° C. (Jagendorf and Avron, 1958). The spinach pulp was filtered through several layers of gauze to eliminate large coarse fibers. The filtrate was then centrifuged at 500 *g* for 30 minutes at 5° C. The supernatant was discarded and the sediment, consisting of almost pure chloroplasts, was reconstituted in 20‰ sea water to desired concentrations.

4. *Injection and sampling procedures*

Seitz-filtered sea water and spinach chloroplast suspension were injected *via* the ventricular route.

Injections were accomplished with a 1.0-ml. tuberculin syringe equipped with a 30-gauge ($1\frac{1}{2}''$) needle. Each oyster received 0.2 ml. of inoculum. There was some leakage immediately following the withdrawal of the needle, but in most cases the wound closed quickly. After injection the oysters were returned to the aquaria until the predetermined sampling intervals had passed.

At the appropriate interval, 0.05 ml. of blood was withdrawn from the ventricle by the same type of syringe and needle used for inoculation. The samples were used for estimating the number of leucocytes and chloroplasts.

5. *Enumeration procedures*

a. Heart rate

The heart was exposed for observation by the method described above. For temperatures above 15° C., the heart rates were calculated on the basis of the time required for 10 beats, while below 15° C., only 3 to 5 beats were used to calculate the number of beats per minute.

b. Unfixed oyster leucocytes

During each sampling 0.05 ml. of blood was withdrawn from the ventricle. Samples were not diluted in most cases; but where dilution was warranted, the sample was diluted with filtered sea water in the syringe and the appropriate dilution factor was noted in calculating the final number of unfixed leucocytes in a given volume of blood. The sample was shaken gently to keep the cells in suspension before the counting chamber was filled. The first two drops of blood expelled from the syringe were discarded. The remaining blood in the syringe was used to fill the improved Neubauer counting chamber by allowing the drops to flow under the cover glass.

c. Fixed oyster leucocytes

The procedure described above did not enumerate all the leucocytes in the sample, for in spite of the agitation of the blood sample, there was still a considerable number of leucocytes adhering to the wall of the syringe. An experiment was designed to compare the fixed and unfixed leucocyte counts from the same blood sample. From each oyster 0.25 ml. of blood was obtained by the method described above. The blood was first used to fill 5 Neubauer counting chambers and the remaining blood was fixed with a 5% acetic acid solution in 20‰ sea water (1 part of blood and 4 parts of fixative). The diluted fixed blood sample was shaken gently to dislodge the adhered leucocytes from the wall of the syringe, from which 5 replicates of the leucocytes were obtained as above.

d. Spinach chloroplasts

In estimation of the concentration of chloroplasts in an inoculum, the suspension was first diluted serially in filtered sea water as follows: 1:10, 1:100, 1:1000 and 1:10,000. The counting chambers were filled with aliquots from the last three dilutions. Counting procedures similar to those for enumeration of oyster leucocytes were used to determine the total number of chloroplasts per ml. of inoculum. For estimating the removal of chloroplasts from the blood stream, the free chloroplasts present were counted simultaneously with the leucocytes in the sample.

e. Statistical treatment of the data

A graphic representation of heart rates and leucocyte counts was derived by calculating the symmetrical confidence limits of these numerical characteristics at the 99% level of probability. Such confidence limits were calculated for a group of control figures by substituting the available information in the following expressions:

$\bar{x} - ts/n$,
where $\bar{x}$ = the arithmetic mean,
t = "Student's" distribution value at the 99% level of significance,
s = standard deviation and
n = the number of observations.

The 99% confidence limits of the control group are plotted. Any point of the experimental groups which falls outside the control limits is considered to be significantly different from the $\bar{x}$ at the 99% level of probability. This method of analysis is the standard procedure used in the study of fluctuations of oyster leucocyte counts, heart rates and chloroplast counts; hence it obviated the requirement of performing many tests of significance between groups of variables.

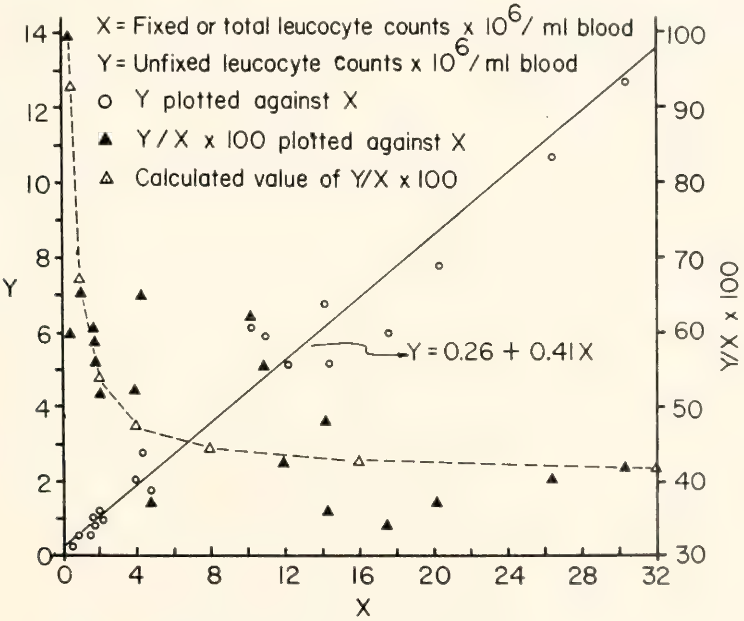

FIGURE 1. Relationship between unfixed and fixed leucocyte counts.

RESULTS

1. *Unfixed leucocyte counts vs. fixed leucocyte counts*

When 20 pairs of unfixed and fixed leucocyte counts are plotted, the points exhibit a linear relationship (Fig. 1). The diagram suggests that an increase in the unfixed leucocyte counts is accompanied by an increase in the fixed leucocyte counts (or the total number of leucocytes). The formula for this regression line is $Y = 0.26 + 0.41X$, where Y is the unfixed leucocyte counts, X is the fixed (or total) leucocyte counts, 0.26 is the intercept of the line with the Y axis, and 0.41 is the slope of the line.

The number of unfixed leucocytes may be expressed as a percentage of the total number of leucocytes ($Y/X \times 100$). When these percentages are plotted against

TABLE I

Variability of leucocyte counts in a group of 88 apparently normal oysters at 18° C.

Leucocyte × 10^6 ml. of heart blood	0.5	2.0	3.5	5.0	6.5	8.0	9.5	11.0	12.5	14.0	15.5	17.0
Frequency	15	20	24	14	3	4	1	2	1	3	0	1
Mean	4.10											
$s(x - x)^2$	1027.83											
Standard Deviation	3.43											
Standard Error	0.37											
n	88											

the total number of leucocytes (X), an exponential curve is obtained (dash line in Fig. 1). The curve suggests that there is little difference between unfixed and fixed leucocyte counts when the total number of leucocytes is in the order of 0.2 × 10^6/ml. blood. Presumably this is due to the fact that the chances of collision between leucocytes and the syringe wall are not great, hence most of the leucocytes remain in suspension. The greatest decrease of the $Y/X \times 100$ term occurs at X values between 0.5 to 4.0 × 10^6/ml. of blood. The adhesion of leucocytes to the syringe wall is probably the greatest in this range, since the chances of contact are enhanced and also the space on the syringe wall does not appear to be restricted. However, further increase in the total number of leucocytes from 4.0 to 30.0 × 10^6/ml. of blood does not seem to affect the percentage term; this is reflected in the flatness of the curve between the above range of leucocyte concentrations. It further suggests that the space of the syringe wall is probably a limiting factor in determining the magnitude of the percentage term.

Based on the above analysis, it is concluded that the unfixed oyster leucocyte count, although not representing the total number of leucocytes is, nevertheless, a valid index of the total number of leucocytes. The data presented in this study are all in terms of unfixed number of leucocytes unless otherwise stated.

2. *Variability of leucocyte counts in apparently normal oysters*

Eighty-eight oysters of assorted sizes were prepared for sampling of heart blood as described above. Prior to the counting of leucocytes, the oysters were removed from the cold room (5° C.) and were placed in sea water of 18° C. as shown in Table I. The range of counts varies from 0.5 × 10^6 to 17.0 × 10^6 per ml. of blood with a mean of $(4.10 \pm 0.37) \times 10^6$ per ml. of blood. Neither sex (Table II) nor wet weight of oysters (3.5–30.1 gm., Fig. 2) is correlated with the number of leucocytes.

TABLE II

Test for the significance of mean leucocyte counts obtained from 33 male and 22 female oysters

Sex	Mean	$s(x - x)^2$	n	SE_D	$x_2 - x_1$	±t	d.f.	*P*
Male	3.23	317.05	33	1.02	0.27	0.26	53	0.5
Female	3.50	225.00	22					

3. *Temperature-heart rate relationship*

Ten partially denuded oysters were placed in sea water at room temperature (23.5° C.), followed by chilling in the cold room. While the water was being chilled, the heart rates were recorded for 23.5°, 21.0°, 18.5°, 14.5°, 11.5° and 10.0° C., respectively. After the observation was completed, the oysters were placed in fresh sea water and kept in the cold room overnight. The next morning, they were allowed to warm up to room temperature in fresh sea water. During the warming process, heart rates were recorded at the above temperature gradient. Three sets of such data were obtained, which were combined to construct the temperature-heart rate curve of Figure 3. The result indicates that the heart rate changes from approximately 5 beats per minute at 10° C. to 29 beats at 23.5° C. and confirms the general conclusion reached by earlier workers (Roughley, 1926; Takatsuki, 1927; Federighi, 1929; and Stauber, 1940).

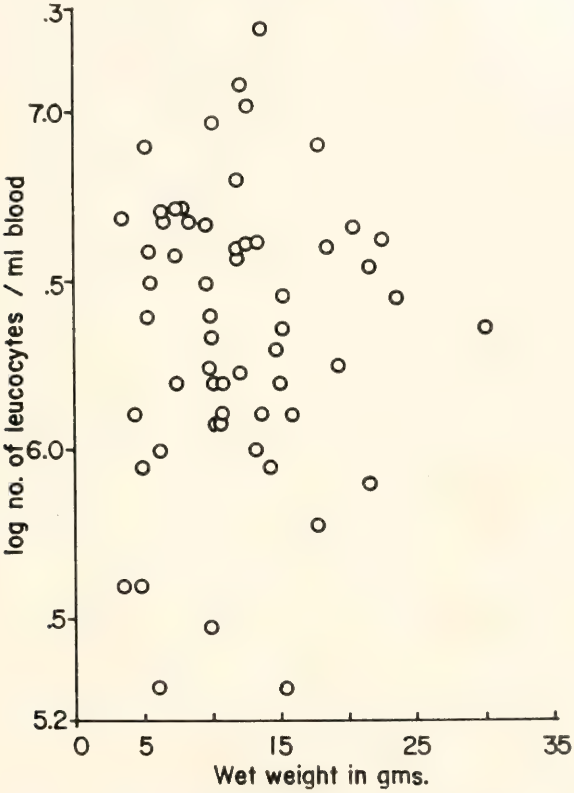

FIGURE 2. A scatter graph of oyster body weights vs. leucocyte concentrations. The plot is based on observations made on 59 oysters.

4. *Temperature-leucocyte number relationship*

Twenty-one oysters were used in this experiment. At 6°, 12°, 18° and 22° C., blood samples were taken from each oyster for the counting of leucocytes. Oysters

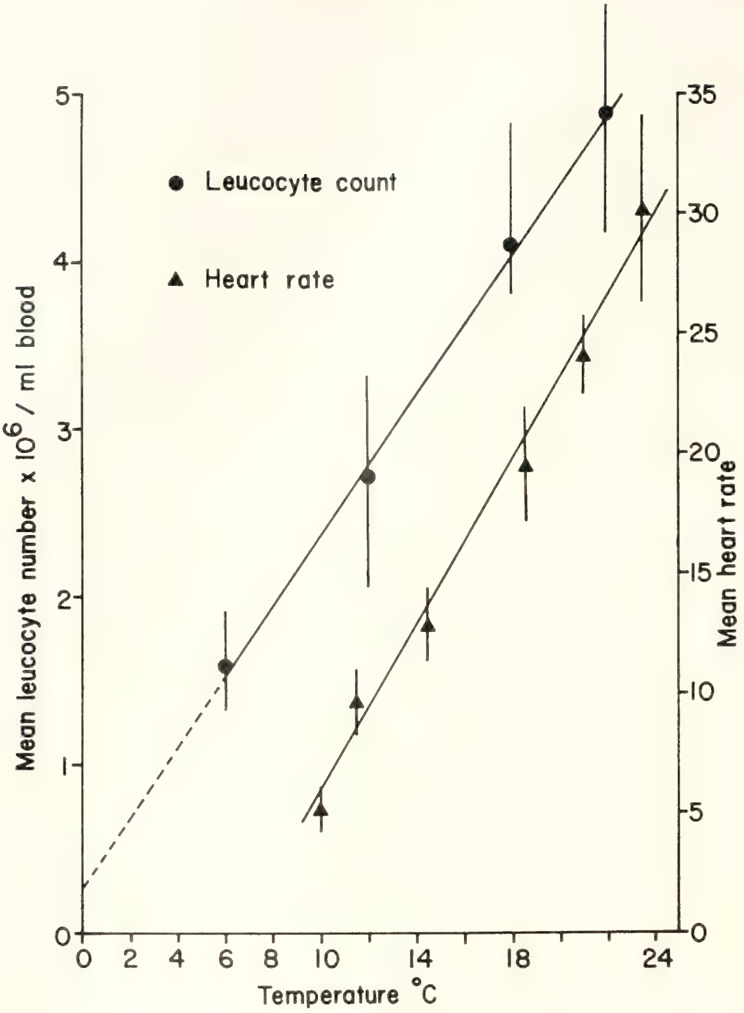

FIGURE 3. The effect of temperature on the leucocyte number and heart rate of oysters. Each point represents average heart rate of three determinations on 10 oysters. The mean leucocyte count in heart blood at 6°, 12°, 18° and 22° C. is obtained from a group of 21 oysters. The vertical lines are ranges of the means.

were allowed to stabilize themselves at each temperature for at least 12 hours prior to sampling.

When the mean number of leucocytes at 6° C. is compared with that at 18° and 22° C., the means behave as if they were drawn from different populations, in spite of the fact that all figures were derived from the same 21 oysters (Table III, *P* less than 0.01). The linearity of the curve (Fig. 3) suggests that the relation between the number of leucocytes in suspension and the temperature change is a simple one. The extrapolated estimate below 6° C. is probably a close approximation to the true event, *i.e.*, leucocytes are nearly 100% settled out at 0° C., since the

TABLE III

Test for significance of mean leucocyte counts obtain from 21 oysters at 6°, 12°, 18° and 22° C.

Temperature ° C.	6°	12°	18°	22°	Remarks
Mean × 10^6	1.06	2.67	4.08	4.86	
$s(x \times \bar{x})^2$	36.31	179.20	217.10	192.57	
Standard Deviation	1.31	2.92	3.22	3.28	
Standard Error	0.29	0.65	0.72	0.68	
n	21	21	21	21	
d.f. = $(n_1 + n_2 - 2)$	—	40	40	40	6° C. *vs.* 12° C.
±t	—	1.49	3.17	4.40	6° C. *vs.* 18° C.
P	—	>0.10	<0.01	<0.01	6° C. *vs.* 22° C.
d.f.	—	—	40	40	
±t	—	—	1.44	2.33	12° C. *vs.* 18° C.
P	—	—	>0.10	>0.02	12° C. *vs.* 22° C.
d.f.	—	—	—	40	
±t	—	—	—	0.80	
P	—	—	—	>0.50	18° C. *vs.* 22° C.

heart is probably quiescent at this temperature. It is possibly true (depending on the length of time) that *in vivo* at 0° C. leucocytes might not be expected to settle out completely due to the increased viscosity of "plasma." However, no leucocytes were found in the supernatant of a blood sample contained in a test tube which was allowed to stand overnight at 25° C. Extrapolation of the temperature-leucocyte relation beyond 22° C. is uncertain; the curve may become asymptotic if the mean of 4.85 × 10^6 leucocytes per ml. blood at 22° C. is the maximum for oysters, or it may rise further if all leucocytic clumping is not yet dispersed or if small foci of leucocytic accumulation in the tissues are resolved.

5. *Effects of mechanical stimuli (repeated bleedings) and of injection of sea water*

This experiment was attempted in order to discover the effects of repeated bleedings in small amounts of 0.05 ml. per bleeding on the number of leucocytes, and to

TABLE IV

Changes in the level of oyster leucocytes during a 20-hour period at 16° C.

Time (hr.)	0	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{3}{4}$	1	3	5	10	20	Grand total
Mean	3.20	4.15	4.00	3.15	3.45	3.65	3.10	2.55	3.40	3.57
$s(x - \bar{x})^2$	29.10	42.03	62.00	73.03	59.73	38.53	24.90	64.23	61.40	463.10
σ	1.71	2.05	2.49	2.70	2.44	1.96	1.58	2.53	2.48	2.27
σ_m	0.57	0.69	0.83	0.90	0.82	0.65	0.53	0.84	0.83	0.24
n	10	10	10	10	10	10	10	10	10	90
d.f.	98	98	98	98	98	98	98	98	98	
±t	0.49	0.76	0.56	0.41	0.16	0.11	0.64	1.32	0.22	
*P**	>0.50	>0.40	>0.50	>0.50	>0.50	>0.50	>0.50	0.20	>0.50	

* Test for significance of the grand total mean and the means obtained at various time intervals.

establish a base line as the control for certain of the studies cited below. Ten oysters held at 16° C. were bled nine times: 0, $\frac{1}{4}$, $\frac{3}{4}$, 1, 3, 5, 10 and 20 hours after the start of the experiment. The grand total mean of leucocytes per ml. of blood based on 90 observations was 3.57×10^6 with a standard error of 0.24 and $s(x - \bar{x}) = 463.1$. The statistical constants of leucocytes obtained at each time interval are summarized in Table IV. Each mean is compared with the grand total mean to ascertain whether the difference between them is significant. The large *P* value, in each case, suggests that the mean number of leucocytes at any time interval during the 20-hour period does not differ significantly from the population mean. The data are also presented in graphic form (Fig. 4).

Concurrently with the above experiment, five oysters were each injected with 0.2 ml. of sea water. Heart blood samples were obtained at the following intervals: $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$, 2, 4, 8 and 96 hours after the injection. Significant changes in leucocyte numbers during the first $1\frac{1}{2}$ hours are observed (Fig. 4). Immediately after the injection, the number of circulating leucocytes drops abruptly; it reaches the lowest point in $\frac{1}{2}$ hour and slowly regains its normal number in about $1\frac{1}{2}$ hours. The sudden drop in leucocyte numbers after the injection of sea water is ascribed to the displacement of oyster blood in the ventricle by the inoculum or to dilution or to disturbance of heart rate or all three together. The gradual mixing of the inoculum with the influx of oyster blood containing many leucocytes results in the subsequent rise of leucocyte numbers. After 2 hours the number of leucocytes in this group

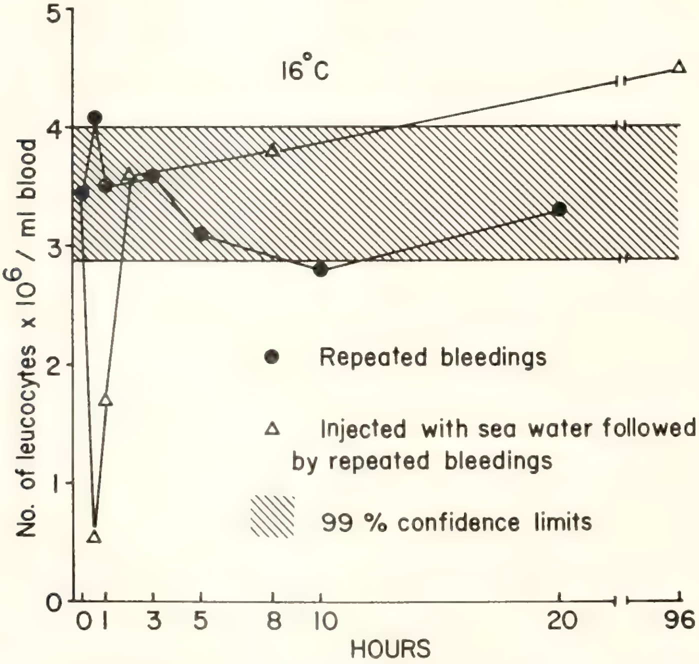

FIGURE 4. The effect of injection of sea water and repeated bleedings on the number of leucocytes in samples of oyster heart blood at 16° C. The cross-hatched area represents the 99% confidence limits of normal leucocyte number in control animals.

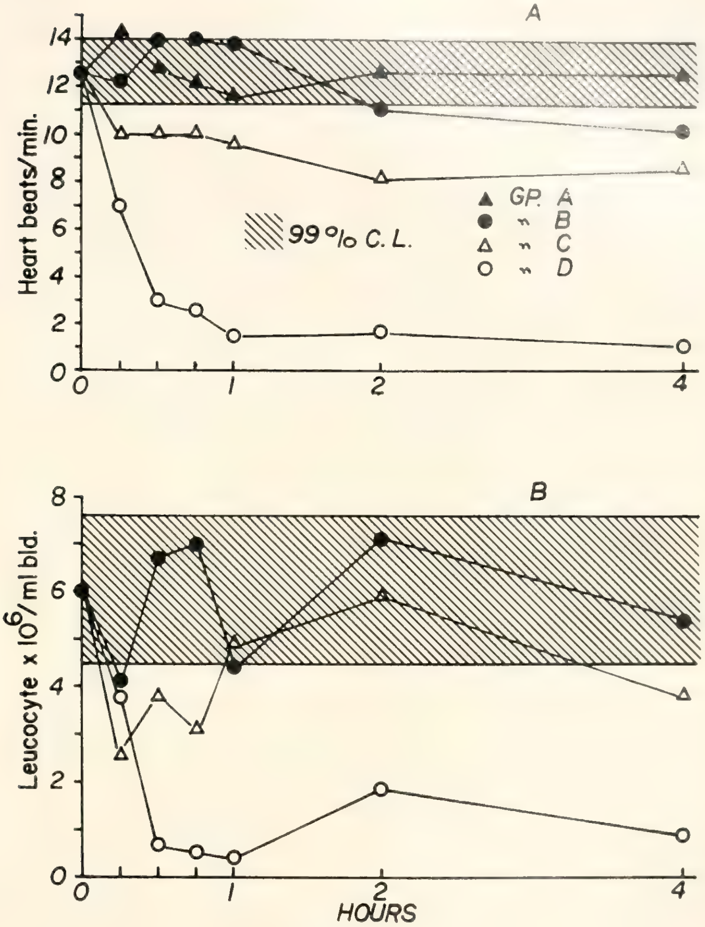

FIGURE 5. The effect of various experimental manipulations on the heart rate (a) and corresponding leucocyte number (b) of oysters. Group A is the control while Groups B, C, and D represent (1) repeated bleedings only, (2) injection of spinach chloroplasts, followed by repeated bleedings, and (3) injection of sea water, followed by repeated bleedings, respectively. The cross-hatched areas represent the 99% confidence limits of heart rates and leucocyte numbers presumed to be present in unhandled oysters and in oysters subject only to repeated bleedings, respectively.

is no longer considered to be significantly different from the control group (Fig. 4), even though one point does fall slightly outside the limits.

6. *Effects of injection of spinach chloroplasts*

Nineteen oysters with hearts exposed were divided into four groups: A, B, C and D. Group A served as control. The remaining three groups were designed to determine the effects of repeated bleedings alone (Group B), injection of sea water followed by bleedings (Group C) and injection of spinach chloroplasts followed by bleedings (Group D) on the heart rate and number of leucocytes. Counts were made on Groups A, B and C at 15°–17° C. with 5 oysters in each group, and on Group D at 14°–16° C. with 4 oysters in the group.

All experiments lasted for 4 hours. Heart rates were recorded for all groups but leucocyte numbers were sampled regularly for Groups B, C and D only. Samples were taken at the following intervals: 0, $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$, 1, 2 and 4 hours. Leucocyte counts were not made for Groups C and D at 0-hour, since 0-hour was also the time when injections were performed. Therefore, it was assumed that the 0-hour leucocyte counts for Groups C and D were probably similar to that of Group B. Each oyster in Group D was given 0.2 ml. of an inoculum containing 6.0×10^9 chloroplasts per ml., while in Group C each oyster was injected with 0.2 ml. of sea water. Ninety-nine per cent confidence limits of heart rates for Group A were calculated and plotted in the manner similar to that for the leucocyte counts.

Repeated bleedings apparently have little effect on the heart rate during the 4-hour period (Fig. 5). Even when the mean heart rate slows down to 8 beats per minute in Group C, which is a significant drop as compared with that of Group A, the leucocyte counts do not show appreciable difference from that of Group B one hour post-injection (Fig. 5). Injections of chloroplasts definitely suppress the heart rates which in turn probably contribute to the delay of the mixing process, among other things. However, the leucopenia immediately following the injection of chloroplasts could be due to incomplete mixing or other unknown factors which are independent of the heart rate.

Discussion

Leucopenia and leucocystosis which are associated with infections in mammals are frequently used as indices of host response; the securing of such information becomes essential in clinical diagnosis. Two parameters may be employed as indices of host responses: (1) changes in ratio of various cells, and (2) quantitative changes in the total number of leucocytes. Although changes in the various types of blood cells after injection of foreign materials or even after hemorrhage were observed by Cameron (1934) for the larvae of the wax moth caterpillar, and also presumably normal leucocyte counts are available for some fishes, amphibians and reptiles (Gemeroy, 1938), the possibility of using this method in detecting host response in poikilothermic vertebrates and invertebrates has yet to be explored. It is probably true that one of the most serious hindrances to the progress is the lack of detailed leucocyte histology with respect to these animals. As a result of the present study, however, it is evident that even the quantitative aspect of this problem is complicated by the fact that the numbers of circulating leucocytes are strongly influenced by the heart rate, which is in turn dictated by temperature and/or mechanical stimuli, *e.g.*, injections of particulate and soluble materials. Thus, the mobilization of leucocytes to the site of invasion in oysters at lower temperatures may be greatly handicapped by the settling out of leucocytes at these low temperatures. The role of the two accessory hearts in circulation has been well established (Hopkins, 1934; Eble, 1963). A comparison of the two accessory hearts and systemic heart with respect to their position, size and mechanical output suggests that the contribution of the former in this context is relatively minor (Eble, 1963; Stauber, personal communication). The available evidence also indicates that they are as temperature-dependent as the systemic heart (Stauber, personal communication). Since leucocytes are also known to participate in the nutritive process, it is possible that the number of circulating leucocytes may fluctuate with stages of feeding, although experimental evidence is still lacking on this point. The transient disappearance of circulating granulocytes following intravenous injection of staphylococci and pneumococci in small mammals (Rogers, 1958) is attributed to adhesion of circulating granulocytes to the capillary endothelium (Wood, Smith, Perry, and Berry, 1951). A similar phenomenon in the oyster, which occurs immediately following intracardial injection of sea water and chloroplasts, is thought to be associated with the incomplete mixing of the inoculum and the influx of blood from the auricles.

Bang (1961) describes the development of "intravascular clotting or thrombosis" in the circumpallial artery of the oyster following intracardiac injection of tissue extracts. He further reports that the "clotting" disappeared spontaneously within two hours after the injection. Based on the findings of the present study, an alternative interpretation of Bang's observations might be offered. It is clearly demonstrated in the present study that the formation and dispersal of leucocyte aggregates or "clotting" can be manipulated by placing oysters at 5° C. followed by gradual warming to 23° C. This observation is further correlated with circulating leucocyte numbers, temperature and heart rate (Fig. 3). The spontaneous disappearance of "clots" within 2 hours after the injection, as reported by Bang, could, therefore, be ascribed to restoration of normal heart rate following recovery of the heart from trauma, since it is the pulsation of the heart which keeps the leucocytes in suspension. It was also not surprising to find that "clotting" did not occur in his control oysters injected with sea water, since in the studies reported here the reduction in heart rate from 13 to 9 beats per minute following injection of sea water (Fig. 5a) did not sufficiently reduce the velocity of blood flow to affect sludging of circulating leucocytes (Fig. 5b). Therefore, what Bang observed in his experimental animals could be a transient sludging of leucocytes induced by temporary heart failure rather than "clotting," which implies the involvement of biochemical processes. Such settling or sludging of blood corpuscles has also been observed in traumatized or sick experimental animals, sick domestic animals and in human patients (Knisely and Bloch, 1942; Bloch, 1956; Harding and Knisely, 1958; Knisely, Warner and Harding, 1960). These authors have clearly demonstrated that the cause of sludging is largely physical, namely, reduction in the velocity of blood flow, created by experimental manipulations, pathological conditions or other factors. The physical aspect of this problem is reflected by the use, in their analysis of the phenomenon, of formulae originally designed for civil engineers in studying the sedimentation rates of various particles in a fluid medium and the transportation of silt by moving water.

The author is deeply indebted to Dr. L. A. Stauber for his many suggestions, helpful criticism and generous support of this work. Without the technical assistance of Mr. Martin Horowitz this study would not have been possible.

SUMMARY

It was demonstrated that the number of circulating leucocytes from the heart blood samples of 21 oysters and the heart rate of oysters increase linearly with the rising ambient temperature at which the oysters were held. At ambient temperatures of 6°, 12°, 18° and 22° C., the mean leucocyte number per ml. heart blood

was found to be 1.6, 2.7, 4.1 and 4.9 million, respectively. The corresponding heart rates were 5 (10° C.), 9, 19 and 26 beats per minute. These findings indicate that the fluctuation of leucocyte counts in the blood of experimental oysters is probably associated with the intensity of agitation exerted by the heart beat, which is in turn influenced by the temperature. Effects of repeated bleedings and of injections of sea water and spinach chloroplasts on heart rate and number of leucocytes were also studied. Repeated bleedings do not affect the heart rate and the number of leucocytes, while injection of sea water and spinach chloroplasts does reduce the heart rate and the leucocyte number significantly for a period of two to at least four hours, depending on the type of inoculum used. These effects, therefore, do influence (1) the settling or sludging of leucocytes, (2) the mixing time of the inoculum and (3) the probable accumulation of leucocytes to the site of invasion, especially in oysters at lower temperatures.

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CHEMOSENSORY BASES OF FOOD-FINDING AND FEEDING IN APLYSIA JULIANA (MOLLUSCA, OPISTHOBRANCHIA)[1]

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While many opisthobranchs have special feeding preferences, the sensory mechanisms involved in their food choices are mostly unstudied (Kohn, 1961; Paine, 1963). *Aplysia juliana*, a common sea-hare of Hawaiian waters, is almost monophagous, feeding only on *Ulva lactuca*, if available, but taking *Ulva fasciata*, if given only this. On the latter, however, the animals do not grow normally. These sea-hares may nibble on other algae, but never eat enough to grow or survive for long. They live well in marine aquaria when fed *Ulva lactuca*, and make good subjects for research on sensory physiology. The animals we tested were maintained in the laboratory in ten-gallon aquaria or one-gallon jars furnished with sub-sand filters. Sea water was obtained locally and used unfiltered. If given food regularly, the animals usually lived for 2–4 months, and grew from a few millimeters to 12–18 cm. long. This report describes the responses of *Aplysia juliana* (hereafter, aplysia) to its food plant, *Ulva lactuca* (hereafter, *Ulva*), with data on sensory processes involved.

RESPONSES TO ULVA

If aplysias are without food for a few days, they usually bury in the sand and remain hidden. One may, on looking into an aquarium, be quite unaware that any of these animals are present. If he drops a small piece of *Ulva* into the water, within 10–15 seconds the oral tentacles of the animals appear, followed by the heads, as the sand seems to come alive, and the aplysias crawl out, with tentacles spread (Fig. 1). They climb the sides of the aquarium, holding fast with the posterior sucker that is characteristic of this species, and extend their tentacles as if sniffing the water, as a dog sniffs the air. If an aerator is in action, the animals may seem not to orient well, but if the water is relatively quiet or moving in a specific direction, they go fairly directly toward the *Ulva*. Upon touching it, they immediately seize it with the mouth and commence feeding.

The response to food occurs, therefore, in three major steps—arousal, orientation, and feeding, as in many predatory and carrion-feeding gastropods (Kohn, 1961). One is impressed, on first seeing the arousal and orientation, by the rapidity and precision of motion for these animals, and the obvious use of the oral tentacles. On contact with the *Ulva*, the aplysias react even more rapidly, as if they were using a different sensory modality. Certain questions immediately come to mind: (1) What is given off by the *Ulva* that attracts the animals? (2)

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With what organ or organs is the stimulus received? (3) How is this behavior related to the general feeding behavior of the animals?

NATURE OF THE ATTRACTIVE SUBSTANCE

It was fairly obvious that only the chemical senses were involved in these reactions. Dropping pieces of other algae or other objects into the water did not arouse quiet animals, thus eliminating mechanical effects. The eyes of these animals would hardly seem to be involved, but to eliminate this possibility the following test was made. A few blades of *Ulva* were put in fresh sea water for about ten minutes, and only the water was then added dropwise to an aquarium holding the aplysias. This proved just as stimulating as the *Ulva* itself. As few as two drops (about 0.2 ml.) of this water added to about 30 liters of sea water in the aquarium—a dilution of one part to 150,000 in terms of water alone; at least 1 in 15,000,000 (assuming the almost certainly high value of 1% dissolved material) in terms of the material—brought the animals forth within 10–15 seconds. Drops of plain sea water, before *Ulva* was added, had no effect when added to the aquaria.

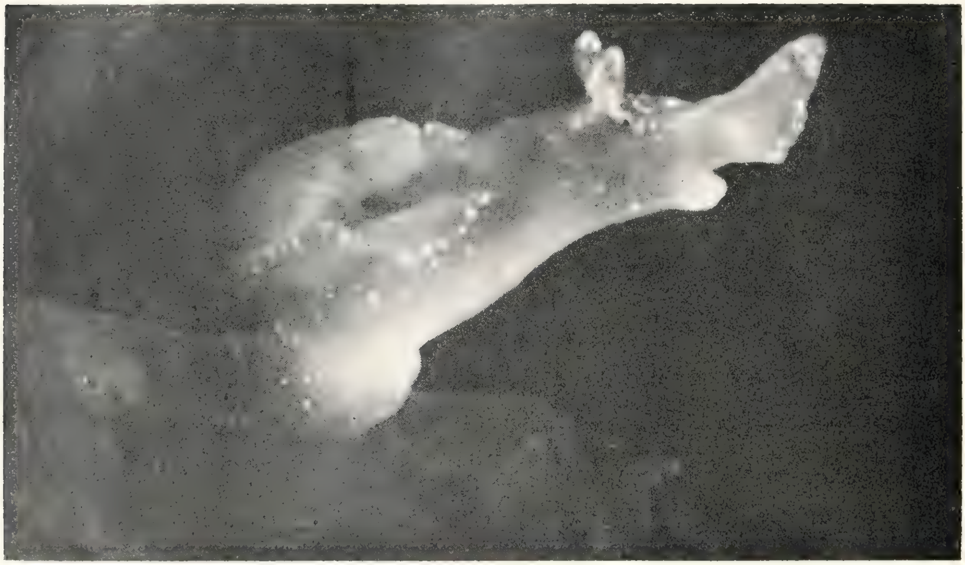

FIGURE 1. Young *Aplysia juliana* (12 mm. long) in presence of material from *Ulva lactuca*. Note spread oral tentacles, posterior sucker of foot, and characteristic seeking posture.

All further experiments were performed with this *Ulva*-water—fresh sea water in which blades of *Ulva* had stood for five or more minutes—for this eliminated other than the chemical senses. Freshly gathered *Ulva* was best for this. If the *Ulva* stood in the laboratory, particularly if in a crowded container, it lost its attractiveness within a few days. Rotting, no matter how little, reduced or abolished the attractiveness.

Preliminary attempts were made to determine the chemical nature of the attractive substance. First, its possible volatility was tested. Air was bubbled through a bottle containing sea water and *Ulva,* and then led through tubing to a bottle containing hungry aplysias. No matter how long or under what circumstances this was done—including varying speeds of air flow, introduction of an air-breaker to break up the air current into bubbles, using small or large quantities of *Ulva,* warming the sea water containing the *Ulva*—the aplysias did not react. In every case, placing a few drops of the sea water covering the *Ulva* into the bottle with the aplysias aroused them almost immediately. *Ulva*-water was boiled for 15 minutes, and then tested. It was as effective as the original solution, as determined by comparative dilution tests—diluting the *Ulva*-water before and after boiling with successive additions of sea water and retesting. Dilutions of five to seven times were possible with both solutions before the effectiveness was lost.

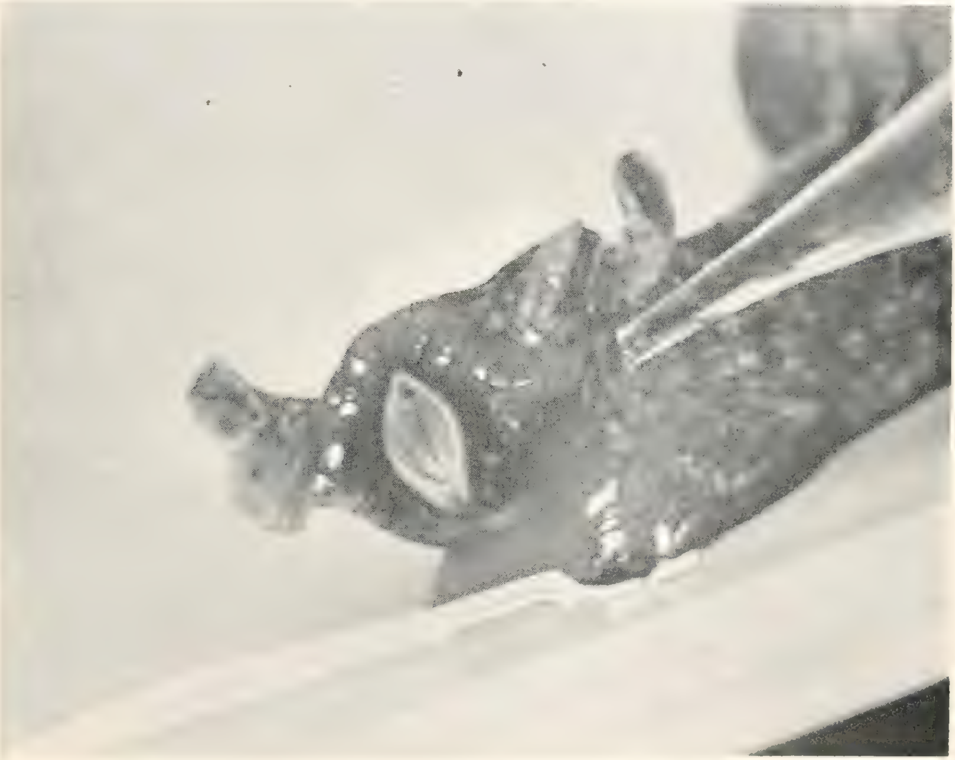

FIGURE 2. Response of *Aplysia* at surface of water to *Ulva*-water dropped from pipette at right onto mouth.

Ether extraction was tried, using a separatory funnel and shaking with ether for 15 minutes. The material remained in the water fraction, and that could be boiled, after the extraction, for 15 minutes and still be as potent as before. The tests so far conducted do not allow much to be said about the chemical nature of the material, except that it seems more like substances associated with contact chemoreception—*i.e.*, non-volatile, water-soluble, heat-stable—where separation of contact and distance chemoreception is possible.

RECEPTIVE AREAS ON THE BODY

To discover the receptive loci for the chemical, *Ulva*-water was allowed to flow gently from a finely drawn pipette onto specific areas of the body of aplysias which had been starved for four or five days. Control tests, using ordinary sea water from a duplicate pipette, were conducted before each experimental test. The following regions of the body were found to be non-receptive: anywhere in the mantle cavity, on the foot, or on the surface of the parapodia, near the anus, the rhinophores, and between the rhinophores and near the eyes. The only areas whose stimulation resulted in orientation and feeding responses were the oral tentacles and the mouth.

The reaction to stimulation of the tentacles is an excellent index of reception. An animal immediately extends the tentacles toward the pipette, then raises its head and reaches for the pipette with its mouth. If the animal is near the water surface, with the foot along the surface, *Ulva*-water can be dropped onto the mouth, and this elicits radular action, as in feeding (Fig. 2). An animal can thus be led around at the surface, with the radula sweeping out regularly. The material, therefore, besides acting as an orienting stimulus, apparently through receptors on the tentacles, also acts as a phagostimulant when applied to the mouth.

The phagostimulant action of *Ulva*-water is surprisingly intense. The aplysias eat filter paper soaked with *Ulva*-water, even though this is much coarser than their ordinary food. Furthermore, they eat other algae, if given *Ulva*-water while the algae are applied to the mouth. Some even try to eat sea anemones that are in the way at the surface, when stimulated by drops of *Ulva*-water on the mouth. If hungry aplysias are mating or laying eggs, drops of *Ulva*-water nearby cause them to stop and to seek the source of the stimulation.

The lack of sensitivity of the rhinophores is worthy of comment, for these organs have been thought by many biologists to be olfactory in function. The name itself implies this, and was so meant (Bergh, 1864). Early comparative anatomists believed that the innervation and cellular structure are like those of the olfactory organs of vertebrates. An olfactory function for the rhinophores is still stated in some general literature, in spite of the reports of Arey (1918), Crozier and Arey (1919), and Agersborg (1922) to the contrary. Stimulation of the rhinophores of aplysias with a current of water brings about turning of the head toward the current if it is gentle, or away if it is strong. The tentacle on the side stimulated is raised and directed toward the current. This reaction is slow enough that it is easy to follow. If the current is of plain sea water, the tentacle ripples through it, then the animal returns to its ordinary behavior. If the current contains the *Ulva*-factor, the animal follows the current as soon as the tentacle intercepts it—but not before. Thus, the rhinophores are sensitive to currents, as Arey and Crozier reported, and the tentacles are chemically sensitive, at least as far as this phagostimulant is concerned. It should be noted that Crozier and Arey, and Agersborg found the rhinophores sensitive to inorganic salts, acids, etc., not to volatile organic compounds, but so were almost all parts of the body. As Kohn (1961) has noted, the relevance of these studies to normal behavior may be questionable.

Discussion of Results

It is obvious that a water-soluble material given off by *Ulva* into sea water acts as a powerful attractant, enabling the aplysias to find food. Either the same or another water-soluble material acts as a strong phagostimulant inducing feeding even on unnatural foods. These act at remarkably low concentrations, dilutions of at least 1 to 15,000,000. The chemical nature of the material or these materials remains to be determined. No attempt can be made, therefore, to designate it or them as contact or distance stimulants (Kohn, 1961).

The receptors for the material acting in arousal and orientation are on the tentacles, and may be confined to these, although receptors in the mouth could be active. The rhinophores are not sensitive to this material, strengthening the conclusions of Arey (1918), Crozier and Arey (1919), and Agersborg (1922) that the name, rhinophore, must not be thought to denote an olfactory function. All critical experimental evidence shows that the rhinophores are primarily receptors for mechanical stimuli, with no more sensitivity to inorganic materials than other parts of the body surface.

Stimulation of the oral chemoreceptors causes feeding behavior. The change in reaction from that elicited by stimulation of the tentacles suggests that a different stimulating chemical or some change in sensory modality may occur. No direct evidence for this was found, however, and it may be that the same material both attracts the animal and stimulates the oral receptors to elicit feeding. The exact receptors were not discovered, but they are near or in the mouth, for stimulation of the mouth alone, without stimulation of the tentacles, elicits feeding reactions.

The induction of feeding on filter paper and other unusual materials by stimulation of the animals with *Ulva*-water suggests that feeding is determined mainly by chemical stimuli. In a few cases, however, some of the animals refuse to feed on filter paper or other cellulose materials, even when flooded with *Ulva*-water, suggesting that mechanical factors also determine food selection.

Observations on Normal Feeding

In captivity, the aplysias generally consume all of the *Ulva* fed to them. Yet, in nature, no matter how many of the animals browse upon a bed of *Ulva*, they do not exterminate it. This observation led us to make some studies on feeding by these animals that indicate why this is so.

Ordinarily, when feeding captive aplysias, we tear *Ulva* from the rocks on which it grows to bring it to the laboratory. If, instead, we bring in rocks with the *Ulva* attached, the aplysias do not completely devour the *Ulva*. Instead they eat the succulent-appearing outer portions of the blades and leave the heavier bases. It is the former that are usually collected when the *Ulva* is merely pulled from the rocks. The basal parts are noticeably coarser (Fig. 3). On examining *Ulva* in regions with high populations of aplysias, we found that the plants that are large enough for feeding obviously have two parts—a basal, darker green, thicker, coarser section, and a distal, succulent, leaf-like portion. Ordinarily, the aplysias eat only the succulent growth; the basal, heavier portions are left. The bases produce new succulent blades within a week or two.

In the aquarium, the same thing is true. The aplysias eat the plants down to the heavy bases, but they do not eat these unless driven to it by long-continued starvation. In many cases, even this does not cause them to eat the bases. The aplysias can be induced to eat the bases by flooding their mouths with *Ulva*-water as they contact the bases. Apparently, if the phagostimulant is present in the basal parts, it is not given off, or is present in too low a concentration to excite the animals.

The ecological significance of this is obvious. By leaving the bases to regenerate new blades, the aplysias do not destroy their food supply completely, even when they become very numerous. They have considerable resistance to starvation, adults living at least two weeks without any food. From an evolutionary standpoint, the situation is quite easily understandable, for the lack of phagostimulant in—or the coarser texture of—the bases saves these for replacement of the blades. The specificity of the aplysias to material from the terminal parts of the blades allows them ordinarily to find plenty of food. The ability of the animals to endure starvation with little weight loss enables them to survive easily during periods when rough seas or other accidents have torn off the edible portions of the *Ulva*. The bases produce new blades rapidly enough to save the animals from death by starvation. The presence or absence of a chemical substance attractive to an animal in different parts of the same plant may thus be responsible for maintaining the food supply of the animal against feeding pressure that would otherwise eliminate the food plant.

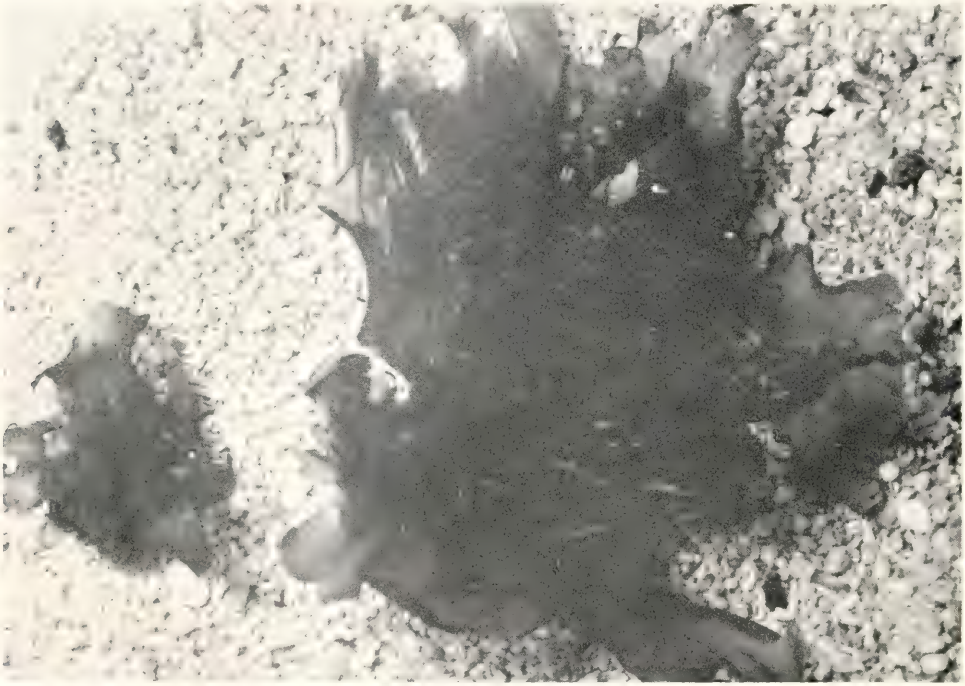

FIGURE 3. *Ulva lactuca*, showing characteristic growth pattern (right), and coarse, basal portion (left) not eaten by aplysias, even though given no other food for several days; the blade was eaten within hours of being given to the animals.

SUMMARY

Sea-hares, *Aplysia juliana*, when hungry, are aroused to activity by and oriented toward *Ulva lactuca*, their normal food. The same response occurs when the animals are stimulated by sea water in which *Ulva* has stood for a few minutes. The material given off by the *Ulva* is non-volatile, heat-stable, and remains in the water fraction after ether extraction. The receptors for this substance, when it acts in arousal and orientation, are on the tentacles, but not on the rhinophores. When sea water in which *Ulva* has stood is dropped onto the mouth of an aplysia, the animal starts feeding activity. The stimulating material acts at very low concentrations and is strongly excitatory. Normally these aplysias eat only the terminal thin parts of *Ulva* and leave the coarser bases which produce new blades. This may be due to the absence of phagostimulant in the bases. The animals therefore do not eliminate the *Ulva*, even when they are very numerous, and their food supply is thus maintained.

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INTRACELLULAR OSMOREGULATION IN SKELETAL MUSCLE DURING SALINITY ADAPTATION IN TWO SPECIES OF TOADS [1]

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The European green toad (*Bufo viridis*) is a surprisingly euryhaline amphibian, some populations living in environmental salinities as high as 29‰ (Stoicovici and Pora, 1951). This survival appears to be largely based upon a tolerance by the tissues of body fluid osmotic concentrations somewhat higher than those of the environment. Changes in osmotic concentrations of body fluids are due primarily (up to 84%) to changes in NaCl content (Gordon, 1962; Tercafs and Schoffeniels, 1962).

The occurrence of large changes in osmotic and salt concentrations in extracellular fluids immediately raises the question of the nature of possible simultaneous changes in intracellular fluid composition. The present paper describes the results of a study of the changes in the principal intracellular solutes in the skeletal muscles of adult green toads adapted to various environmental salinities. For comparison, similar data are presented for the less euryhaline western American toad, *Bufo boreas*. A preliminary account of some of the results of this work was given by Gordon (1963).

MATERIALS AND METHODS

Adult green toads were collected on the island of Saltholm, near Copenhagen, Denmark, in May, 1962, and were shipped by air to Los Angeles. In Los Angeles they were maintained at 19 ±2° C. in tap water dechlorinated with a charcoal filter. They were fed twice weekly with larval and adult *Tenebrio*. Survival was excellent. Some toads not used in experiments remained alive and in good condition for more than two years. No attempt was made to separate the sexes.

Adult western toads (*Bufo boreas*) were obtained as needed from local suppliers in southern California. Maintenance conditions were as for green toads.

Toads were adapted for two or more days to dilutions of natural sea water in groups of six or more in an air-conditioned room maintained at 19 ±2° C. Sea water dilutions (100% sea water = 32‰ salinity = 950 mOsm/l. osmotic concentration) were made with dechlorinated tap water. Higher salinities were ap-

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TABLE I

Analytical procedures

Analysis	Procedure	Plasma sample vol. (μl.)	Average precision of dupl. analyses	Muscle sample wt. (mg.)	Average precision of dupl. analyses	Reference
Osmolarity	Freezing point depression	0.001	±20 mOsm/l.	—		Ramsay & Brown (1955)
Total solids	Wet-dry weights	—	—	100–200	±2 gm./kg.	Gordon (1962)
Chloride	Ag^+ titration	50	± 3mEq/l.	100–200	±1 mEq/kg.	Cotlove (1963)
Sodium	Flame photometer	100	±3 mEq/l.	100–200	±0.5 mEq/kg.	Gordon (1962); for muscle water homogenates of fresh samples.
Potassium	Flame photometer	100	±0.2 mEq/l.	100–200	±2 mEq/kg.	Gordon (1962); for muscle, water homogenates of fresh samples.
Non-protein N (NPN)	Nesslerization	50	±20 mg./l.	100–200	±0.03 gm./kg.	Natelson (1961)
α-NH_2N	Na β-naphthoquinone-4-sulfonate	100–200	±2 mg./l.	100–200	±0.02 gm./kg.	Frame *et al.* (1943); Russell (1944)
Urea	Urease, microdiffusion	25	± 10 mg./l.	100–200	±0.01 gm./kg.	Conway (1957a); for muscle as for sodium above
Inulin	Resorcinol	100	±30 mg./l.	100–200	±0.001 gm./kg.	Roe *et al.* (1949); see text
Total sugar	Anthrone		–	50–70	±0.2 gm./kg.	Seifter & Dayton (1950)
Glycogen	Anthrone		–	50–70	±0.2 gm./kg.	Seifter & Dayton (1950)
Free amino acids (qualitative)	2-dimensional paper chromatography	100	—			Allen & Awapara (1960); Scherbaum *et al.* (1959); see text
Free amino acids (quantitative)	Spinco Model 120 amino acid analyzer	—	—	1000–1500	see text	Spackman *et al.* (1958)

proached in graded steps (*e.g.*, successive three-day periods in fresh water, 20%, 40% and 50% sea water). Toads were not fed during salinity adaptation.

Blood samples were obtained by exposing and opening the heart, then collecting the blood in lightly heparinized glass capillary tubes. These tubes were closed with

TABLE II

Inulin spaces in toads adapted to various salinities

State of adaptation	$[\bar{x} \pm S.E. (N)]$	
	Total extracellular fluid (l./kg. body wt.)	Muscle extracellular fluid (l./kg. wet wt.)
Bufo viridis		
FW	0.418 ± 0.019 (5)	0.128 ± 0.036 (5)
20% SW, 7–15 days	0.398 ± 0.019 (4)	0.139 ± 0.035 (3)
40% SW, 5–13 days	0.364 ± 0.014 (5)	0.132 ± 0.060 (4)
50% SW, 2–6 days	0.338 ± 0.022 (5)	0.175 ± 0.011 (5)
Bufo boreas		
FW, start	0.332 ± 0.026 (4)	0.116 ± 0.035 (5)
FW, starved 1 mon.	0.331 ± 0.019 (3)	0.120 ± 0.072 (3)
20% SW, 7–15 days	0.365 ± 0.020 (5)	0.139 ± 0.038 (3)
40% SW, 2–8 days	0.288 ± 0.037 (5)	0.164 ± 0.034 (5)
50% SW, 3 days	0.270 (1)	0.159 (1)

sealing wax, centrifuged and resealed after the packed red cells were cut away. Plasma samples were stored frozen at −20° C. for later analysis.

Samples of skeletal muscle ranging in weight, as required by the experiment, from 50 to more than 1000 mg. were taken from the thighs. Muscle samples were subdivided and the pieces weighed to ± 0.2 mg. on a torsion balance. Prepared muscle extracts were stored frozen at −20° C. for later analysis.

Procedures for sample preparation and analysis are summarized in Table I. Some additional information is necessary for two procedures.

Inulin spaces: Inulin spaces ("extracellular spaces") were determined both for the whole animal and for thigh musculature. Toads were weighed to ±0.1 g., then injected intraperitoneally with 0.5 ml./100 g. of 10% inulin in 0.5% NaCl solution. Pilot studies on both species showed that plasma inulin concentrations reached a plateau stable for at least four hours within two hours of injection.

TABLE III

Plasma concentrations in toads adapted to various salinities
[$\bar{x}$ ± S.E. (N)]

State of adaptation	Osmolarity (mOsm/l.)	Cl^- (mEq/l.)	Na^+ (mEq/l.)	K^+ (mEq/l.)	NPN (mg./l.)	Free $\alpha-NH_2N$ (mg./l).	Urea N (mg./l.)
Bufo viridis							
FW	279 ±13 (5)	99 ±2 (5)	113 ±4 (5)	4.7 ±0.4 (5)	1040 ±110 (5)	46 ±4 (10)	970 ±150 (5)
20% SW 7–15 days	290 ±10 (5)	114 ±3 (5)	126 ±2 (5)	5.8 ±0.3 (5)	920 ±40 (5)	45 ±3 (10)	790 ±40 (5)
40% SW 5–13 days	441 ±8 (5)	159 ±5 (5)	173 ±4 (5)	6.5 ±0.3 (5)	1980 ±130 (5)	70 ±6 (12)	1930 ±150 (4)
50% SW 2–6 days	502 ±9 (5)	196 ±1 (5)	207 ±2 (5)	10.0 ±0.8 (5)	2090 ±60 (4)	78 ±5 (9)	1920 ±60 (5)
Bufo boreas							
FW start	156 ±22 (5)	61 ±8 (5)	64 ±25 (4)	4.1 ±0.2 (5)	580 ±110 (5)	58 ±6 (9)	630 ±160 (4)
FW starved 1 mon.	—	—	—	—	—	39 ±3 (6)	—
20% SW 7–15 days	253 ±15 (4)	102 ±2 (5)	98 ±11 (5)	4.8 ±0.2 (5)	590 ±60 (5)	57 ±3 (10)	420 ±50 (5)
40% SW 2–8 days	366 ±15 (3)	172 ±4 (4)	183 ±2 (3)	5.9 ±0.1 (3)	1150 ±40 (3)	88 ±8 (10)	850 ±130 (4)
50% SW 3–6 days	—	—	—	—	—	84 ±6 (3)	—

Plasma and muscle samples were taken from injected toads 2–6 hours after injection. Paired control samples were taken from similarly treated uninjected animals. Following analysis, mean background values for inuloid substances in the muscles were subtracted from the measured inulin values in injected toads. This background was usually quite constant within groups treated in a given way. No background correction was necessary for plasma. There was always close agreement between the muscle inulin spaces measured in the two thighs of individual animals.

Muscle free $\alpha-NH_2N$ and free amino acids: Muscle samples for these analyses were taken immediately after sacrifice of the experimental animal. They were weighed on a torsion balance and homogenized in ice-cold 10% trichloroacetic acid (for $\alpha-NH_2N$), 80% ethanol (for paper chromatography) or 1% picric acid (for the amino acid analyzer). Checks on possible rapid production of amino acids by hydrolysis of tissue proteins were made for the first two methods by doing

TABLE IV

Muscle concentrations in toads adapted to various salinities

[All amounts per kg. wet wt.; $\bar{X} \pm$ S.E.(N)]

State of adaptation	Total solids (gm.)	Cl^- (mEq)	Na^+ (mEq)	K^+ (mEq)	NPN (gm.)	Free α-NH_2N (gm.)	Urea N (gm.)	Total sugar (gm.)	Glycogen (gm.)
Bufo viridis									
FW	210±4(10)	24±1(5)	34±1(5)	64±1(5)	3.7±0.2(5)	0.94±0.02(10)	0.83±0.15(5)	10.1±0.7(5)	9.6±0.9(5)
20% SW 7–15 days	211±5(10)	27±2(5)	32±2(5)	65±2(5)	3.3±0.1(5)	1.00±0.03(10)	0.88±0.06(5)	—	
40% SW 5–13 days	249±5(12)	57±4(5)	68±4(5)	66±2(5)	4.6±0.1(5)	1.31±0.06(12)	1.82±0.18(5)	7.3±1.1(5)	6.7±1.3(5)
50% SW 2–6 days	281±4(9)	70±4(5)	78±5(5)	72±2(5)	4.9±0.1(5)	1.48±0.06(9)	1.76±0.09(5)	—	
Bufo boreas									
FW start	208±5(9)	20±2(5)	27±2(5)	75±6(5)	2.8±0.3(5)	0.71±0.05(9)	0.68±0.14(5)	7.8±0.4(5)	7.2±0.1(5)
FW starved 1 mon.	210±3(6)			—	—	0.70±0.03(6)	—	—	
20% SW 7–15 days	194±2(10)	27±3(5)	37±3(4)	77±3(5)	3.0±0.1(5)	0.85±0.02(9)	0.56±0.06(5)		
40% SW 2–8 days	270±6(10)	49±7(4)	62±8(4)	86±11(4)	3.8±0.1(4)	1.22±0.04(10)	1.15±0.09(4)	10.5±1.0(5)	9.5±1.0(5)
50% SW 3–6 days	276±8(3)	—	—	—	—	1.39±0.09(3)	—	—	

parallel analyses on duplicate aliquots of tissue which were either allowed to remain at room temperature for several minutes before being homogenized or were homogenized in the appropriate solutions at room, rather than ice, temperatures. No statistically significant differences were found. These results agree with those of Roberts and Simonsen (1962).

Mean intracellular concentrations for substances in the muscles were calculated using the following equation:

$$[A]_i = \frac{[A]_m - [A]_p v_e}{[H_2O]_m - v_e}$$

$[A]_i$ = mean intracellular concentration of A
$[A]_m$ = mean wet muscle concentration of A
$[A]_p$ = mean plasma concentration of A
v_e = mean muscle inulin space
$[H_2O]_m$ = mean wet muscle water content

The most variable quantity involved in these calculations is v_e. Estimates of the variability in $[A]_i$ produced by variation in v_e were obtained by recalculating $[A]_i$ using v_e ±2 S.E.'s. The variability figures given in Table V are the average deviations about the means, calculated by this procedure.

These calculations were made on the assumption that there are no large differences between plasma concentrations and concentrations in muscle extracellular fluid. The data of Conway (1957b) and Maurer (1938) support this assumption

TABLE V

Intracellular concentrations in muscles of toads adapted to various salinities (all amounts per kg. cell water; cf. text for method of calculation)

State of adaptation	Cl (mEq)	Na^+ (mEq)	K^+ (mEq)	NPN (gm.)	Free α-NH_2N (gm.)	Urea N (gm.)
Bufo viridis						
FW	17 ± 8	30 ± 10	95 ± 10	5.4 ± 0.5	1.42 ± 0.01	1.07 ± 0.01
20%, SW 7–15 days	17 ± 10	22 ± 11	98 ± 10	4.9 ± 0.4	1.52 ± 0.01	1.18 ± 0.04
40%, SW 5–13 days	58 ± 20	73 ± 20	105 ± 20	7.0 ± 1.0	2.10 ± 0.02	2.54 ± 0.11
50%, SW 2–6 days	66 ± 6	77 ± 5	130 ± 6	8.4 ± 0.3	2.68 ± 0.16	2.61 ± 0.02
Bufo boreas						
FW start	19 ± 4	30 ± 4	110 ± 10	4.0 ± 0.4	1.04 ± 0.20	0.90 ± 0.02
FW starved 1 mon.	—	—	—	—	1.03 ± 0.02	—
20% SW 7–15 days	19 ± 10	34 ± 8	114 ± 12	4.3 ± 0.4	1.26 ± 0.01	0.75 ± 0.04
40%, SW 2–8 days	37 ± 16	57 ± 15	150 ± 18	6.4 ± 0.6	2.14 ± 0.03	1.78 ± 0.12
50% SW 3–6 days	—	—	—	—	2.44 ± 0.05	—

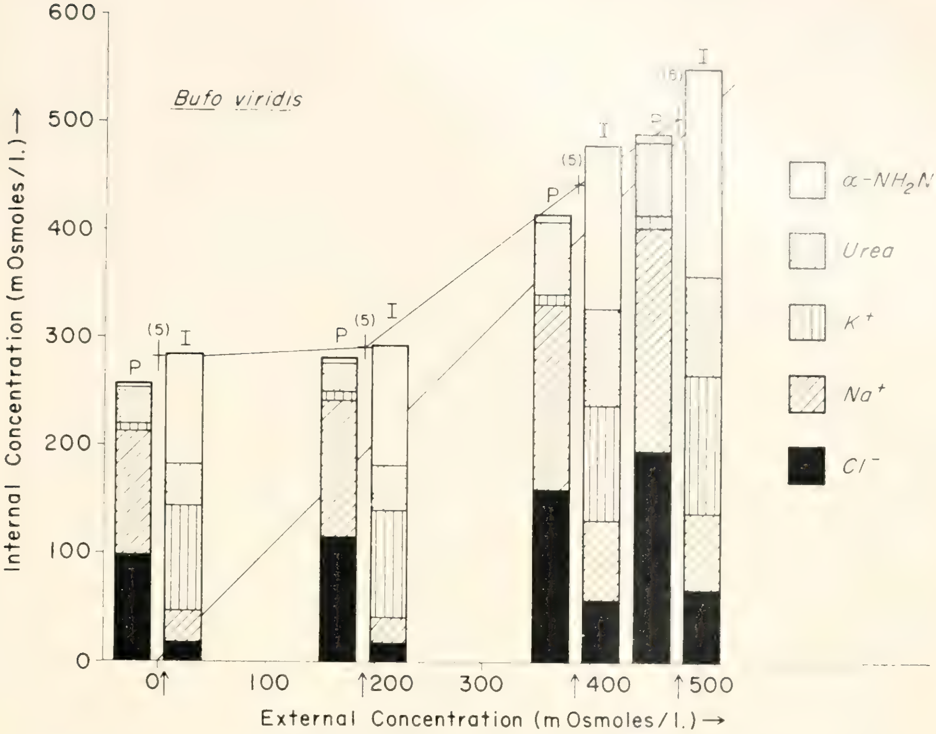

FIGURE 1. Composition of plasma (P) and intracellular fluid (I) in skeletal muscles of *Bufo viridis* acclimatized to fresh water, 20%, 40% and 50% sea water (arrows along abscissa indicate acclimatization concentrations). Data from Tables III and V. Upper continuous line, plasma osmotic concentration; second continuous line, equality between internal and external concentrations. Vertical lines on each point ±2 S.E.'s of means. Left-hand bar in each pair plasma concentrations; right-hand bar calculated intracellular concentrations.

for frogs. Sodium concentrations were not corrected for possible sarcolemmal binding (Conway and Carey, 1955; Dee and Kernan, 1963).

RESULTS

Whole body and muscle inulin spaces in both *Bufo viridis* and *Bufo boreas* adapted to various concentrations of sea water are listed in Table II. There are differences between the two species with respect to whole body space, but no such differences for muscle space. *B. viridis* had whole body inulin spaces 3–8% larger than those found in similarly adapted *B. boreas*. Neither salinity adaptation nor starvation for a month significantly affected whole body spaces in *B. boreas*. In *B. viridis*, however, whole body space decreased by 7% (significant at 5% level by "t" test) between fresh water and 50% sea water.

The maximum environmental concentration tolerated indefinitely by *B. viridis* was 50% sea water. *B. boreas* survived well in 40% sea water, but died within a week in 50% sea water. Whole animal and muscle inulin spaces in one *B.*

boreas, after three days in 50% sea water, were insignificantly different from those of normal-appearing, active toads in 40% sea water.

Muscle inulin spaces in both species are in the range usually reported for amphibian skeletal muscle. There were no statistically significant variations in muscle inulin spaces in differently adapted animals in either species. Variability between animals was fairly large, however, so possible small differences may be masked.

The results of analyses of plasma and muscle samples taken from variously adapted *B. viridis* and *B. boreas* are presented in Tables III and IV. These basic data have been combined with the muscle inulin space data of Table II to yield estimates of intracellular concentrations in the skeletal muscle fibers of the two toads. These latter estimates are presented in Table V. Figures 1 and 2 compare the major features of these sets of data.

Both forms maintain osmotic concentrations of the plasma at levels higher than medium concentrations until maximum salinity tolerances are reached. Increases in plasma concentrations above those characteristic of fresh water are due primarily to NaCl (86% of the increase in *B. viridis* between fresh water and 50% sea water; 109% of the increase in *B. boreas* between fresh water and 40% sea water—

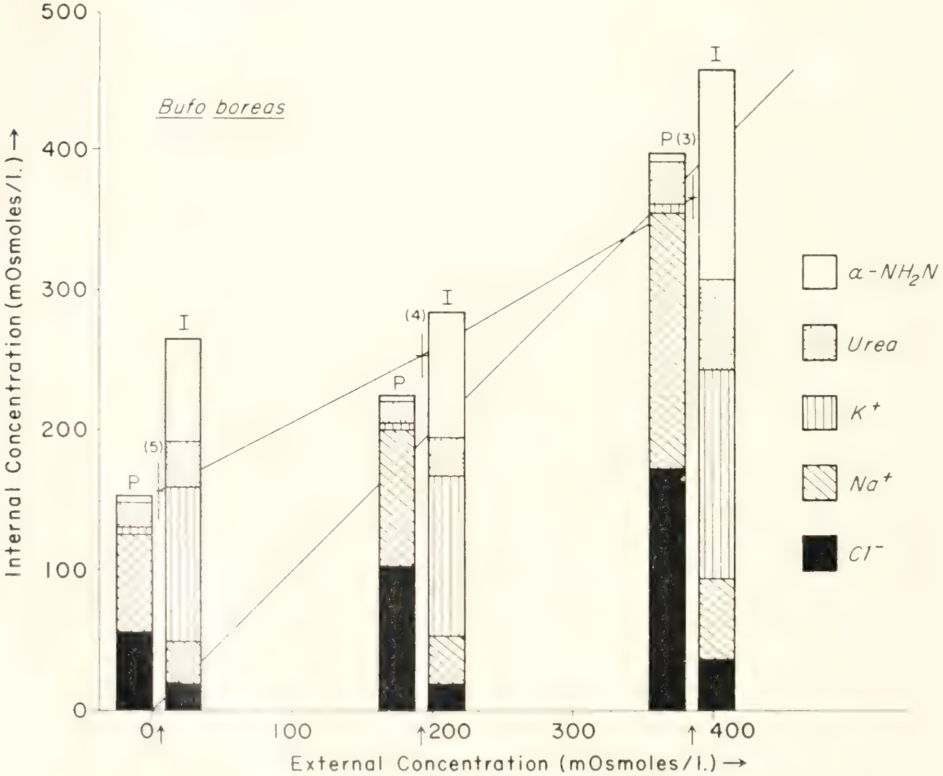

FIGURE 2. As Figure 1 but for *Bufo boreas.* Toads acclimatized to fresh water, 20% and 40% sea water.

a mathematical anomaly due primarily to the variability of the osmolarity measurements). Changes in plasma urea concentrations account for only a small fraction of the osmolarity changes (14% in *B. viridis* between fresh water and 50% sea water; 4% in *B. boreas* between fresh water and 40% sea water). Plasma amino acid concentrations are low (3–6 m*M*) in both forms in all salinities. In all salinities plasma urea N plus α-NH_2N account for essentially all plasma NPN in *B. viridis* and for 81% or more of plasma NPN in *B. boreas.*

Plasma osmolarity increased 80% in *B. viridis* between fresh water and 50% sea water, and 135% in *B. boreas* between fresh water and 40% sea water. Muscle solids in the same groups of animals, however, increased by only 33% in both species. The muscles, therefore, maintained great stability in water content in the face of major changes in extracellular osmotic concentration. Since muscle inulin spaces were constant, there were only minor decreases in fiber volumes. The main phenomenon was adjustment of intracellular concentrations so as to minimize hydration changes.

The calculated intracellular concentrations indicate that all measured cellular solutes contributed to these adjustments. The major contributions in both forms came from the inorganic ions, urea, and free amino acids and related compounds. No significant amounts of free carbohydrates were present in the muscles of either species.

Rough estimates of total intracellular osmotic concentrations may be obtained from the data in Table V by calculating the millimolar equivalents for α-NH_2N and urea N, then adding these to the inorganic ion data. One may then calculate the fractional contributions made to changes in total estimated intracellular osmotic concentrations by changes in the various components.

For *B. viridis* between fresh water and 50% sea water, chloride and sodium each accounted for 17%, potassium for 13% of the estimated total change. These three ions together accounted for about 47% of the change. Free amino acids accounted for about 33%, urea for the remaining 20% of the estimated change.

For *B. boreas* between fresh water and 40% sea water, chloride and sodium, respectively, accounted for 9% and 14%, potassium for 20% of the total intracellular change. These ions together accounted for 43% of the change. Free amino acids comprised 40%, urea 17% of the estimated difference.

In all states of salinity adaptation, intracellular urea concentrations in both species apparently exceeded plasma urea levels. The ratio $\text{urea}_{\text{in}}/\text{urea}_{\text{out}}$ was variable, ranging from 1.10 to 1.50 in *B. viridis,* from 1.42 to 2.10 in *B. boreas.* If intracellular urea is not bound, as is usually assumed (Bozler, 1961), these ratios would indicate significant urea accumulation by toad muscle fibers.

Urea and amino acids accounted for almost all of the changes in intracellular NPN which occurred. Starvation for one month produced no change in intracellular free α-NH_2N in *B. boreas.*

The finding of large changes in muscle content of free amino acids raised the question of which amino acids were involved. Muscle samples were taken from five toads of each species adapted to fresh water and from five toads of each species adapted to 40% sea water. Separate one- and two-dimensional paper chromatograms were run of acidic and basic amino acid fractions from each sample. These chromatograms demonstrated: (1) Major amino acid changes were restricted to a few amino acids, there being differences between the two species in the specific compounds affected. (2) The pattern of major changes was constant within a given species.

Preliminary quantitative estimations of specific amino acids which changed were made by column chromatographic analyses of muscle samples from one toad each of each species in fresh water and 40% sea water. Ninhydrin-positive compounds found in this way are listed in Table VI. Only components attaining concentrations greater than 2 mM/kg. in at least one animal are listed.

TABLE VI

Major ninhydrin-positive compounds in muscles of toads

Compound (mM/Kg. wet wt.)	State of adaptation			
	Bufo viridis		*Bufo boreas*	
	FW	40% SW	FW	40% SW
Glycerophosphoethanolamine	2.8	3.3	1.1	4.2
Phosphoethanolamine	5.1	5.9	2.8	4.4
Taurine	19	28	8.7	23
Urea	43	84	20	59
Aspartic acid	0.3	2.5	—	—
Serine	0.6	2.6	—	—
Asparagine-glutamine	1.0	5.4	—	—
Glutamic acid	3.3	5.4	2.1	14
Glycine	2.1	12	5.0	2.7
Alanine	0.2	8.9	1.7	3.0
Creatinine	—	—	3.8	0
Carnosine	1.8	3.6	0.4	11

Considering only those compounds which changed in concentration by more than 5 mM/kg. wet weight, important increases occurred in *B. viridis* for taurine, urea, glycine and alanine. In *B. boreas,* similarly large increases occurred for taurine, urea, glutamic acid and carnosine. These changes were probably the only ones which were statistically significant, based on experience with column chromatographic analyses of replicate muscle samples taken from other amphibian species.

DISCUSSION

Bufo viridis and *Bufo boreas* appear to be the first vertebrates known to use large quantities of free amino acids in intracellular osmoregulation. The hagfishes (Myxini) also have high blood salt concentrations, but no more than 10–15% of intracellular osmotic concentrations in these forms are due to free amino acids. Intracellular free amino acid concentrations in *Myxine glutinosa* in 100% sea water are only 60–70 mM/kg. fiber water (Bellamy and Jones, 1961). This level is about one-third that found in both species of toad adapted to only 50% sea water. Other species of toads apparently normally maintain about 100 mM/kg. fiber water of intracellular free amino acids, even in the absence of hydration stresses (Briner, 1961).

Free amino acids have long been known to be of importance in intracellular osmoregulation in many groups of invertebrates (recent reviews include Awapara, 1962; Florkin, 1963; Kittredge *et al.*, 1962; Lockwood, 1962).

There is as yet no experimentally defined relationship between the changes in intracellular solute composition described and the changes in intracellular osmotic concentration which occurred. Intracellular osmotic concentrations probably change with and are equal to simultaneous extracellular osmotic concentrations. While this situation has not yet been conclusively proven (Robinson, 1960, for review), data such as those of Buckley *et al.* (1958) and Bloch *et al.* (1963) seem convincing. However, all cell water may or may not be available to solutes (Dydyńska and Wilkie, 1963; Lindenberg and Gary-Bobo, 1960). Intracellular sodium activity may be quite low (Lev, 1964). Very little is known of the degree to which "free amino acids" are actually free in solution, or whether other substances, such as urea, may not actually be partially bound (Bozler, 1961; Heinz, 1962).

The differences in the nature of the compounds changing in the two species presumably are reflections of metabolic differences between the two forms. It is probable that equally large metabolic differences exist among the various tissues within each species (Roberts and Simonsen, 1962). The chemical origins of the substances changing in concentration are unknown. Changes in external ionic environment have been shown to produce important metabolic shifts in isolated muscles of *Bufo marinus* (Muller, 1962).

The change in carnosine content in *B. boreas* may be accounted for in several ways. Severin *et al.* (1962) indicate that carnosine may play a role in neuromuscular transmission in frogs. They found that the carnosine content of pieces of muscle containing many motor end plates was significantly higher than the carnosine content of pieces containing few or no end plates. The present results may thus: (1) be related to the changing needs of intracellular osmoregulation; (2) indicate an important change in neuromuscular function in *B. boreas* associated with salinity stress; or (3) be simply a sampling error due to differences in numbers of motor end plates included in the muscle samples analyzed.

The present data on muscle inulin spaces agree well with some previous determinations of this quantity in amphibian muscle (Boyle *et al.*, 1941; Dee and Kernan, 1963; Simon *et al.*, 1957). They are significantly lower and less variable than other results (Steinbach, 1961; Tasker *et al.*, 1959). These latter groups of higher and more variable results may be due to handling procedures used in experiments involving soaking of isolated muscles in Ringer solutions of various types (Dydyńska and Wilkie, 1963).

The present data on plasma composition in *B. viridis* agree with previous results (Gordon, 1962). Both sets of data are quite different from those of Tercafs and Schoffeniels (1962). The reasons for the disagreement are not apparent.

SUMMARY

1. A detailed study of intracellular osmoregulation in skeletal muscles has been carried out in two species of toads, *Bufo viridis* and *Bufo boreas*, adapted to various external salinities between fresh water and 50% sea water (salinity 16‰).

2. Both species are osmoconformers, changes in plasma osmotic concentrations being due almost entirely to changes in NaCl concentrations. Muscle dry weight,

however, is more stable, increasing by less than 35% in both forms in the face of osmotic concentration increases of 80–135% in the plasma. Muscle extracellular volume (inulin space) is constant, independent of changes in plasma concentration.

3. Relative stability of muscle hydration is due to accumulation of intracellular solutes. Changes in intracellular osmotic concentration are broadly partitioned: 47% inorganic ions (Cl, Na, K), 33% free amino acids and related compounds, 20% urea in *B. viridis;* 43% inorganic ions, 40% free amino acids and related compounds, 17% urea in *B. boreas.* Free carbohydrates appear to be virtually absent.

4. Increases in intracellular free amino acids and related compounds involve different substances in the two species. *B. viridis* accumulates primarily taurine, glycine and alanine. *B. boreas* accumulates primarily taurine, glutamic acid and carnosine. Intracellular urea concentrations seem always to be significantly higher than plasma concentrations.

5. Uncertainties in the data, related primarily to lack of knowledge of the physical state of intracellular solutes, are discussed. Various implications of the results are considered.

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THE ROLE OF CREATINE AND ITS PHOSPHATE IN AMPHIBIAN DEVELOPMENT[1]

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Baldwin (1957) has summarized the chemical events that occur in a normal muscle working under anaerobic conditions:

(1) Adenosine triphosphate (ATP) remains unchanged;
(2) Phosphocreatine (PC) disappears;
(3) Free creatine (C_F) appears;
(4) Free inorganic phosphate (P_I) appears;
(5) Glycogen disappears;
(6) Lactic acid appears.

The classical theory of muscle contraction accounts for these occurrences by means of assumptions schematized in Figure 1. As long as it is active, the muscle undergoes endergonic changes of state, as shown on the right. The necessary input of energy is provided by the dephosphorylation of ATP, converting it into ADP (adenosine diphosphate) and liberating free P_I. Part of the ADP thus formed is immediately phosphorylated by PC, thus regenerating some ATP as fast as it is utilized and elevating somewhat the level of C_F. The remainder, together with free P_I, stimulates glycolysis, which in the absence of oxygen terminates in lactic acid, and which helps to relieve the drain on stored PC by generating further ATP. The imbalance finally reached is a measure of the discrepancy between the rate at which energy is demanded by the contractile process and the rate at which it can be supplied by glycolysis.

An energy transfer and storage system similar to that operative in muscle is believed to occur in amphibian embryos, and in the sequel some of the evidence will be reviewed. However, the role of creatine and phosphocreatine has never been thoroughly investigated, although it has been hinted at by the data of Zielinski (1937) and of Barth and Jaeger (1947). Thus, there has been a considerable hiatus in our knowledge of the major features of the energetics of amphibian development.

The present investigation is, in part, a study of energy transfer and storage in *Rana pipiens* embryos, with special attention to the role of phosphocreatine under both aerobic and anaerobic conditions. Also, advantage has been taken of the opportunity to conduct a parallel study of gastrula-arrested hybrid embryos obtained by fertilizing *R. pipiens* eggs with *R. sylvatica* sperm (Moore, 1941, 1946; Gregg, 1957). Direct estimation of free and total creatine has made possible the calculation of the amounts of phosphocreatine phosphorus (PCP) present at vari-

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[1] This paper is based on a thesis submitted to Duke University in partial fulfillment of the requirements for the degree of Master of Arts.

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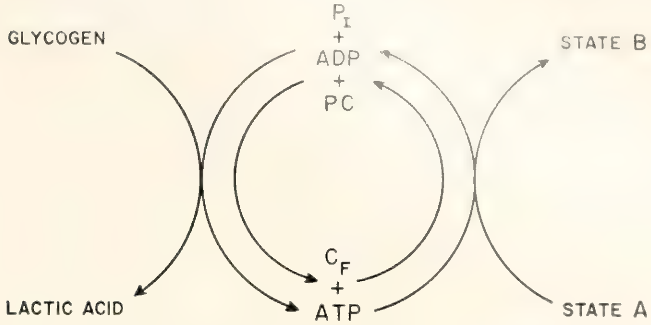


FIGURE 1. Chemical events occurring in muscle contracting anaerobically.

ous stages, aerobically and anaerobically, and this has been compared with directly estimated free inorganic phosphorus. Total phosphorus (P_T) and total acid-soluble phosphorus (P_A) have also been estimated as checks against the possibility of artifacts arising from phosphorus loss to the surrounding medium; and further guarantee that the embryos have been maintained within physiological bounds has been sought in a study of the ability of *R. pipiens* embryos to develop normally after anaerobic treatment.

I thank Dr. John R. Gregg for suggesting this problem, for his encouragement and helpful criticisms throughout the work, and for his assistance with the manuscript.

METHODS

Embryological

Embryos were obtained by stripping eggs from pituitary-treated *R. pipiens* females into suspensions of *R. pipiens* or *R. sylvatica* sperm. After 30 minutes, the sperm suspensions were poured off and fresh 10% amphibian Ringer's solution (without phosphate or bicarbonate) was added. After an additional 30 minutes, the embryos were separated into small groups and allowed to develop, as convenient, at 10° C., 18° C. or at room temperature (21° C.). Before subjecting the embryos to anaerobiosis, their jelly coats were removed with jewelers' forceps. Aerobic embryos were dejellied just prior to chemical analysis.

Developmental stages, and the corresponding standard ages at 18° C., were ascertained by reference to Shumway (1940).

Anaerobiosis

The apparatus used to provide an anaerobic environment is represented in Figure 2. Dejellied embryos (125) were placed in the bottom of the chamber, along with enough 10% Ringer's solution to immerse the tip of the gas inlet tube. After tipping the chamber so that the inlet tube cleared the surface of the medium, pre-purified nitrogen (oxygen content less than 45 p.p.m.) was flushed rapidly through the chamber for one minute. The chamber was then clamped vertically in a rack, and lowered partly into a water bath maintained at 24° C. Nitrogen was allowed to bubble slowly through the medium for 10 minutes, after which all stopcocks were closed.

Aerobic control embryos were placed in unstoppered Erlenmeyer flasks in the same water bath.

Protein-free extracts

After a prescribed time in the chamber, the embryos were quickly staged, transferred into chilled graduated centrifuge tubes, washed with ice-cold deionized distilled water, made up to a volume of 5 ml. with water, decanted into a chilled glass homogenizer containing 1.25 ml. of 24% trichloroacetic acid and homogenized. After taking an aliquot for the determination of total phosphorus, the homogenate was centrifuged at 0° C. for 10 minutes at 5000 *g*. The clear supernatant was then pipetted into a chilled test tube and kept ice-cold to prevent breakdown of labile phosphates.

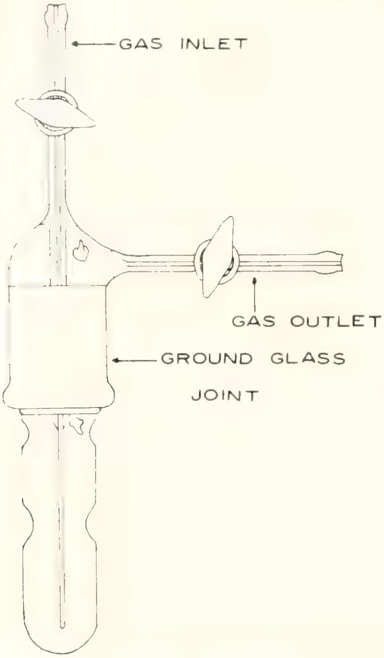


FIGURE 2. Diagram of nitrogen chamber.

Aerobic control embryos were treated simultaneously in exactly the same manner.

Chemical analysis

Creatine moieties were determined by means of the method of Ennor and Rosenberg (1952), slightly modified. In this procedure, creatine is estimated colorimetrically before (C_F—free creatine) and after (C_T—total creatine) acid hydrolysis, and the difference $C_B = C_T - C_F$ is assumed to be the bound creatine of phosphocreatine.

A portion of the trichloroacetic extract was neutralized with powdered Na_2CO_3 (Kutsky, 1950), and two 0.5-ml. aliquots were transferred into separate test tubes. One aliquot (C_F) was set aside; the other (C_T) was acidified with 0.25 ml. of 0.3 N HCl, hydrolyzed for 30 minutes in a water bath at 56° C., cooled and re-neutralized with 0.25 ml. of 0.3 N NaOH. Two ml. of freshly prepared diacetyl reagent were then added to each tube. The tubes were placed in the dark, to prevent a photoreaction between trichloroacetate and alpha naphthol, and incubated for 35 minutes at room temperature. Optical densities at 525 mμ were determined with a Bausch and Lomb Spectronic 20 colorimeter. A blank and two standards (creatine hydrate) were run simultaneously.

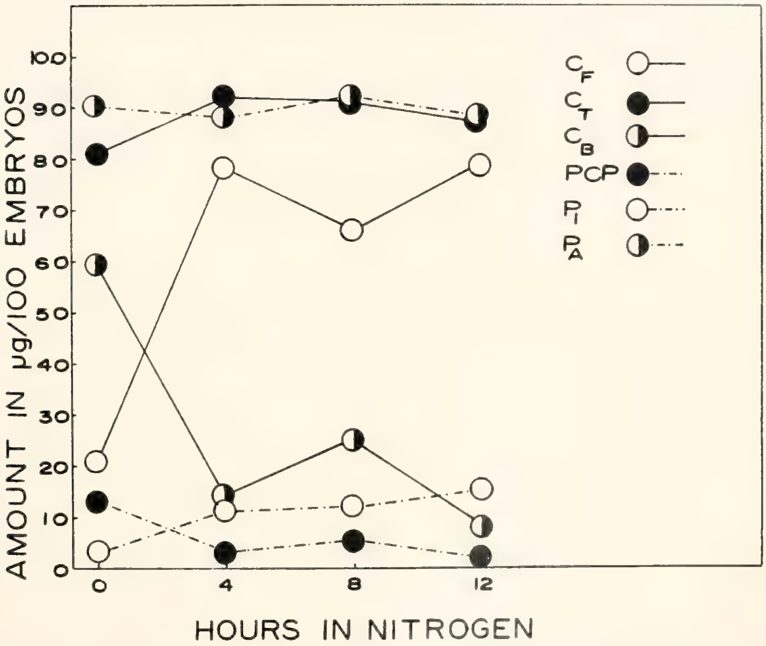


FIGURE 3. Changes in creatine and phosphorus fractions in *Rana pipiens* embryos at Stage 10-, after various anaerobic periods.

The amount of phosphocreatine phosphorus was calculated, once the value of C_B was known, from the equation $PCP = 0.2362\ C_B$, where the numerical constant is the value of the ratio of the molecular weight of phosphorus to the molecular weight of creatine.

Inorganic phosphorus was determined by the method of Berenblum and Chain (1938), as modified by Martin and Doty (1949). A 2-ml. aliquot of protein-free extract was placed in a test tube, followed by 5 ml. of 1:1 benzene:isobutanol and 1 ml. of molybdate reagent. The tube was stoppered with a ground-glass stopper and shaken vigorously for 15 seconds. When it had formed, 2.6 ml. of the upper phase were transferred with a syringe into a centrifuge tube and made up to 5 ml. with acid alcohol. Stannous chloride reagent (0.5 ml.) was then added, and after 20 minutes the optical density at 625 mμ was determined with the colorimeter mentioned above. A blank and two standards (KH_2PO_4) were run simultaneously.

The method of Martin and Doty (1949) was also used for the determination of total phosphorus and total acid-soluble phosphorus, but with the modifications suggested by Ernster *et al.* (1950). Two test tubes were prepared, one (total phosphorus) containing 0.2 ml. of homogenate, the other (total acid-soluble phosphorus) containing 1 ml. of protein-free extract. After the addition of 1 ml. of 10 N H_2SO_4 to each tube, they were placed in an oven at 130–160° C. After two to three hours, and after partial cooling, one drop of 30% H_2O_2 was added to each tube, which was then heated over a bunsen burner until the contents became colorless or very pale yellow in color. The tubes were then replaced in the oven for 15 minutes. After partial cooling, 1 ml. of water was added to each tube, which was then returned to the oven, now at 100° C., for 10 minutes. The tubes were then cooled, and the contents made up to 7 ml. with water. Inorganic phosphorus present was then determined as described above, with the exception that H_2SO_4 was omitted from the molybdate reagent.

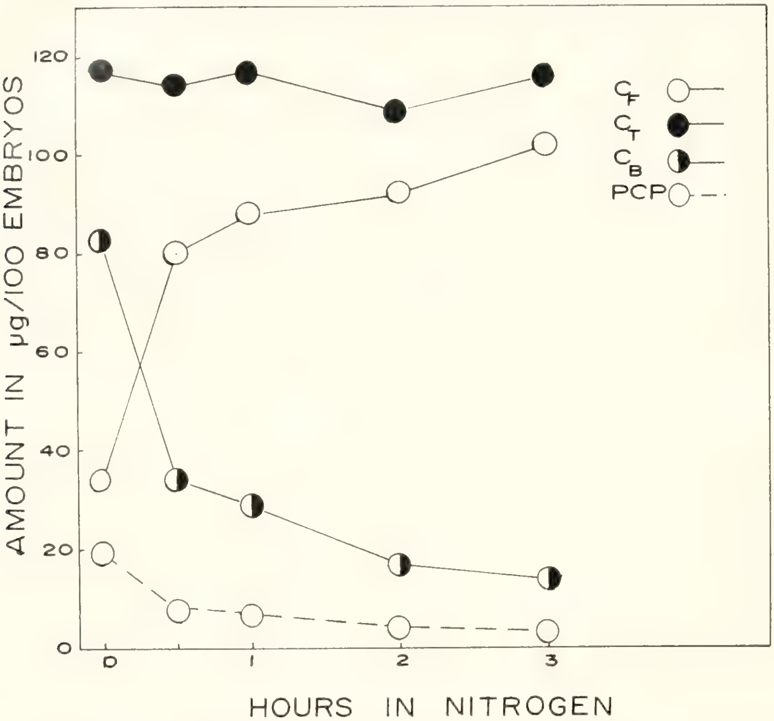


FIGURE 4. Changes in creatine and phosphorus fractions in *Rana pipiens* embryos at Stage 11 after short exposure to anaerobiosis.

RESULTS AND DISCUSSION

Preliminary experiments

In order to avoid working with irreversibly damaged material, an attempt was made to ascertain how long embryos (*R. pipiens*) at various stages could be kept anaerobic at 24° C. and still develop normally when aerobiosis was resumed. It turned out that late-cleavage embryos and gastrulae are less susceptible to permanent damage by anaerobiosis than embryos of other stages, and will survive a sojourn of 12 hours in nitrogen; but two hours of anaerobiosis is the maximum that will permit subsequent normal development of embryos in *all* stages from fertilization to hatching.

TABLE I

Creatine and phosphorus moieties of R. pipiens and R. pipiens ♀ × R. sylvatica ♂ embryos after 2 hours of anaerobiosis at 24° C., in micrograms per 100 embryos

Treatment	Range of stages	*R. pipiens*					Hybrids					No. expts.
		C_T	PCP	P_I	P_A	P_T	C_T	PCP	P_I	P_A	P_T	
Aerobic Anaerobic	3–4	94.4 93.8	14.0 7.0	2.2 5.3	90.0 91.2	2071 2068	90.6 91.6	12.6 6.7	2.4 6.1	89.0 94.0	1929 1911	5
Aerobic Anaerobic	9–11	94.8 95.8	14.4 2.9	3.7 11.8	87.6 90.0	1998 2081	91.7 92.0	13.8 2.3	3.3 11.3	88.8 88.8	1926 1944	6
Aerobic Anaerobic	12–14	93.8 102.4	12.1 3.7	5.1 15.6	85.2 91.0	2002 2062	93.2 95.2	12.0 2.8	2.6 12.1	88.2 87.4	1936 2139	5
Aerobic Anaerobic	15–16	94.7 91.7	13.4 2.4	5.1 18.5	84.3 89.3	2055 2029	86.7 87.3	11.4 3.1	3.1 12.4	80.7 83.3	1987 1947	3
Aerobic Anaerobic	17–18	109.0 108.0	15.4 1.7	7.4 21.4	100.5 107.0	1905 1931	94.3 91.8	11.4 1.4	4.2 13.8	93.7 90.7	1914 2104	4
Aerobic Anaerobic	19–20	145.0 145.0	19.3 2.5	10.2 35.5	128.3 134.3	1946 1922	86.0 83.0	9.9 2.1	6.6 17.5	96.0 92.0	1851 1888	3

Having established this result, two experiments were made to determine the rapidity with which creatine and phosphorus imbalances are established in *R. pipiens* embryos (gastrulae) under anaerobiosis. The results (Figs. 3 and 4) indicate that the major changes occur during the first hour in nitrogen, although some further change is evident throughout the 12-hour period examined.

As a consequence of the foregoing, two-hour periods of anaerobiosis were adopted as standard for further experimentation; and it is believed that this guarantees comparability of the results obtained with embryos of different ages—and of the results obtained with normal and hybrid embryos, although tests of the nitrogen-tolerance of hybrid embryos were not made.

Results obtained by other investigators indicate that temperature, length of anaerobiosis and embryological type all affect the extent of the phosphorus imbalance induced by oxygen-deprivation. Barth and Jaeger (1947), in a study of

R. pipiens and *R. pipiens* ♀ × *R. sylvatica* ♂ gastrulae, found that free inorganic phosphorus reached a maximum level between 4 to 8 hours after the onset of anaerobiosis and that this level was essentially maintained up to 22 hours (18° C.). Little change in the level of ATP was observed in either type of embryo; and this agrees with the results of Brachet (1954), who examined *Bufo vulgaris* and *B. vulgaris* ♀ × *R. fusca* ♂ embryos subjected to 8-hour periods of anaerobiosis at 12° C. In the case of embryos produced from eggs (*R. fusca*) fertilized with nitrogen-mustard-treated sperm (*R. fusca*), Brachet (1954) observed a 60% decrease of ATP after 8 hours anaerobiosis at 20° C., as compared with a 30% decrease in normal anaerobic controls. In another experiment at the same temperature, embryos of both types lost 62% of their ATP after 17 hours of anaerobiosis.

TABLE II

Comparison of various phosphorus fractions

Species	μg. Pi per 100 embryos	μg. PCP per 100 embryos	μg. Cr per 100 embryos
R. temporaria (Zielinski, stage unspecified)	6.5 (2.9–11.5)	8.2 (3.8–11.4)	104 (85–143)
R. pipiens (Harrison, stage 13)	5.1 (2.8–7.3)	12.1 (11.0–14.8)	94 (78–111)

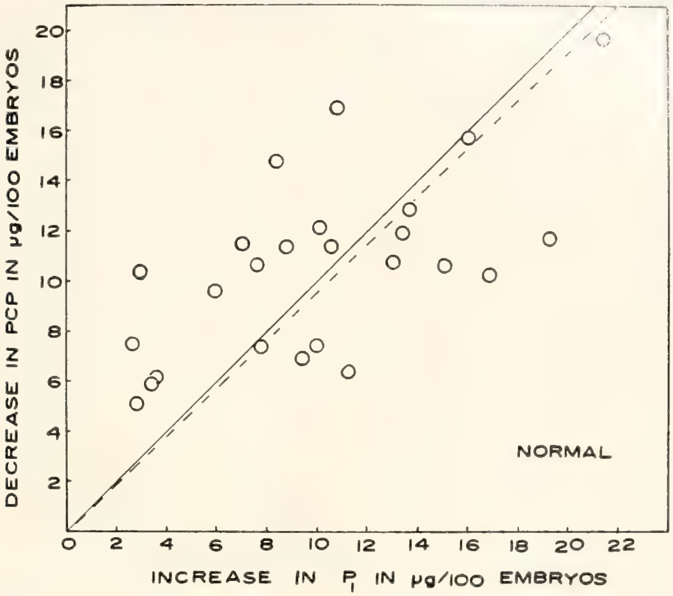
Creatine and phosphorus moieties

The results of all further chemical analyses are summarized in Table I, whose significance will be elaborated in the following discussion.

As functions of the developmental stage of aerobic *R. pipiens* embryos, total phosphorus is constant, free inorganic phosphorus is steadily increasing, and total acid-soluble phosphorus, phosphocreatine phosphorus and total creatine are approximately constant before stage 16 but increasing thereafter. As regards total phosphorus, free inorganic phosphorus and total acid-soluble phosphorus, aerobic hybrids exhibit roughly the same developmental changes, though to a lesser extent in respect of the latter two fractions; while phosphocreatine phosphorus declines in amount after stage 16, and total creatine is constant throughout.

For *R. pipiens*, it may be noted that the aerobic levels of inorganic phosphorus, phosphocreatine phosphorus, and total creatine approximate those reported for *R. temporaria* by Zielinski (1937), although his methods have been questioned on (unstated) technical grounds by Barth and Jaeger (1947). A comparison of the two groups of results is set out in Table II.

In general, the values for inorganic phosphorus in aerobic *R. pipiens* embryos agree more closely with those obtained by Kutsky (1950) than with the higher values obtained by Barth and Jaeger (1947) and by Gregg and Kahlbrock (1957). Among other things, this may be the result of using a chemical method that reduces the breakdown of labile phosphates by shortening their exposure to acid molybdate reagent. As compared to hybrid embryos, the higher levels of free inorganic phosphorus developed by normal embryos suggested to Gregg, MacIsaac and Parker (1964) that they operate at a relative energy-deficit; but in view of the fact that the increases are more than balanced by increasing total acid-soluble phosphorus, it seems better to look upon them merely as a reflection of higher steady-



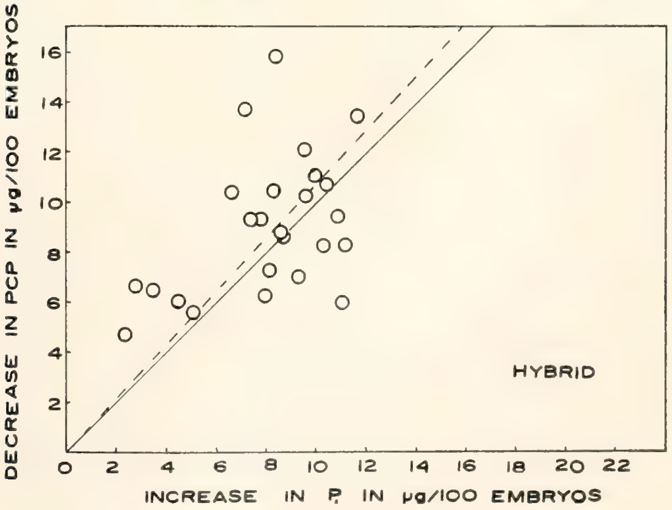


FIGURE 5 (above). Ratio of change in phosphocreatine phosphorus to change in inorganic phosphorus in *Rana pipiens* embryos during a two-hour anaerobic period. The solid line is the theoretical regression line and the dotted line the calculated regression line.

FIGURE 6 (below). Ratio of change in phosphocreatine phosphorus to change in inorganic phosphorus in *Rana pipiens* ♀ × *Rana sylvatica* ♂ hybrid embryos during a two-hour anaerobic period. The solid line is the theoretical regression line and the dotted line is the calculated regression line.

state levels. This interpretation is reinforced by the subnormal total creatine levels of post stage 16 hybrids, which may place some limitation on the amount of energy that can be stored as phosphocreatine.

Under anaerobiosis (two hours), total phosphorus, total creatine and total acid-soluble phosphorus remain relatively stable in both types of embryos. Phosphocreatine phosphorus, however, undergoes a marked depletion, which is almost exactly balanced by the appearance of free inorganic phosphorus (Figs. 5 and 6). This may be taken to indicate that no change of ATP-level occurs, as in muscle. Under longer periods of anaerobiosis (12 hours), more free inorganic phosphorus appears than can be accounted for by the disappearance of phosphocreatine

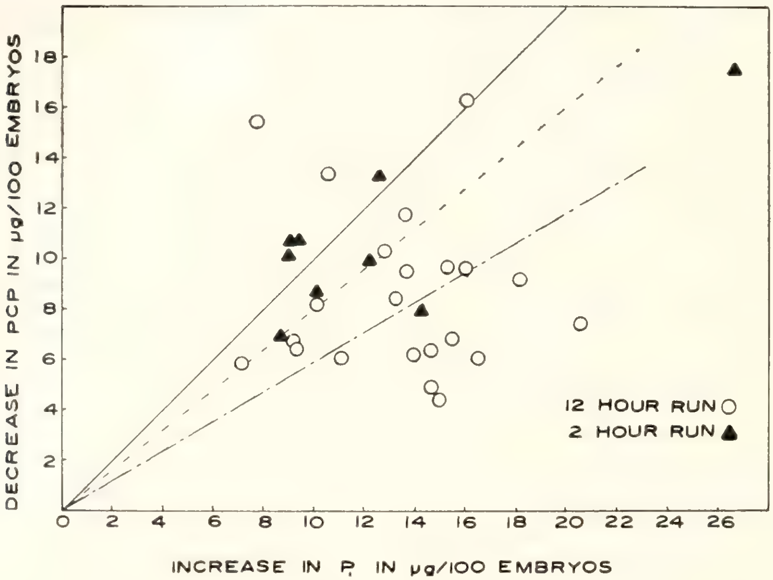


FIGURE 7. Ratio of change in phosphocreatine phosphorus to change in inorganic phosphorus during anaerobiosis in *Rana pipiens* embryos. The solid line represents the theoretical line on which points should fall if the ratio is 1:1. The dotted line is the calculated regression line for the two-hour runs and the dotted-dashed line is the calculated regression line for the 12-hour runs.

phosphorus, at least in normal embryos (Fig. 7), perhaps owing to the breakdown of ATP. These observations are in agreement with the results of Carlson and Siger (1957), who found that net phosphocreatine breakdown in iodoacetate-poisoned frog sartorius muscle is a linear function of work done, and that no net breakdown of ATP occurs until the phosphocreatine concentration drops by 60% or more. They also account for the findings of Barth and Jaeger (1947) that during long periods of anaerobiosis (10–22 hours), more inorganic phosphorus appears than can be attributed to ATP breakdown, in both normal and hybrid embryos.

It should be noted that the phosphocreatine phosphorus-inorganic phosphorus imbalance induced by anaerobiosis is much more pronounced in normal embryos than in hybrids, at least in older embryos. This is readily understandable in terms of the relatively greater energy-demands imposed by the complexity of the developmental changes occurring in normal embryos, in comparison with those of lesser complexity occurring in blocked hybrids.

Taken all together, the foregoing results suggest that the role of the creatine moieties in the energetics of amphibian development is the same as in that of contracting muscle: the phosphorylation of creatine provides a store of phosphate bond energy that may be drawn upon in periods of anaerobic stress. Indeed, in view of the demonstration of a phosphorylating anaerobic glycolysis in amphibian embryos (Cohen, 1954), the parallel between muscle and embryos appears to be nearly complete.

We may now add a few remarks about the role of energy metabolism in the development of blocked hybrids. Post-blockage hybrids have subnormal respiratory and glycolytic rates (Barth, 1946; Gregg, 1960, 1962), and utilize stored glycogen at a rate lower than normal (Gregg, 1948). Some ultimate limitation upon their capacity to generate and store energy is indicated by their subnormal respiratory response to the uncoupling agent 2,4-dinitrophenol (Gregg, 1960) and by their failure to synthesize creatine after stage 16, as mentioned in the discussion above. Nevertheless, homogenates of hybrid embryos exhibit respiratory and glycolytic rates that are quantitatively similar to those of normal embryos (Gregg and Ray, 1957; Gregg, MacIsaac and Parker, 1964), and the general picture of energy production, transfer and storage that emerges is one of essential normality, in which actually occurring energy-demand is met without undue difficulty. Thus, the lowered rates of energy metabolism may be regarded as a reflection of some independent block, internal or imposed by the embryonic environment, to developmental transformations that would normally require a greater expenditure of energy.

SUMMARY

1. A study has been made of the role of phosphorylated creatine in the energy metabolism of *Rana pipiens* and gastrula-blocked *R. pipiens* ♀ × *R. sylvatica* ♂ hybrid embryos.

2. It is shown that the longest period of anaerobiosis that can be survived by *R. pipiens* embryos of all pre-hatching stages, with subsequent normal aerobic development, is of two hours duration, at 24° C.

3. In hybrid embryos, creatine synthesis is deficient after stage 16, and the levels of phosphocreatine phosphorus and inorganic phosphorus are subnormal.

4. In both types of embryos, two hours of anaerobiosis result in a decrease of phosphocreatine phosphorus, accompanied by a quantitatively similar increase of free inorganic phosphorus. Both changes are greater in normal embryos than in hybrids. In *R. pipiens* embryos, prolonged anaerobiosis (12 hours) results in the appearance of excess inorganic phosphorus, perhaps at the expense of ATP.

5. A comparison is made of energy metabolism in muscle and embryos, and the role of energy metabolism in the development of hybrids is briefly discussed.

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AN AUTORADIOGRAPHIC INVESTIGATION OF THE GONADS OF THE PURPLE SEA URCHIN (STRONGYLOCENTROTUS PURPURATUS)[1]

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The duration of the gametogenic events following DNA synthesis by primary spermatocytes and primary oocytes may be determined autoradiographically following the administration of tritiated thymidine. Primary spermatocytes and primary oocytes in premeiotic DNA synthesis are the most advanced germ cells that can incorporate tritiated thymidine. Therefore, by labeling such cells with tritiated thymidine and preparing autoradiograms of gonad samples taken at increasing time intervals thereafter, the labeled cells may be followed as they differentiate into gametes. It is assumed that, before the appearance of labeled gametes, the most advanced labeled cells in any gonad sample have been derived from labeled primary oocytes or primary spermatocytes, and not from labeled gonial cells. By this method, the latter part of oogenesis has been timed in mice (Lima-de-Faria and Borum, 1962; Rudkin and Griech, 1962), while the latter part of spermatogenesis has been timed in rats (Clermont *et al.*, 1959), fruit flies (Chandley and Bateman, 1962), and man (Heller and Clermont, 1963). In the present investigation, the method has been used to study oogenesis and spermatogenesis in the purple sea urchin, *Strongylocentrotus purpuratus*, a marine invertebrate having separate sexes and an annual reproductive cycle. Gonads from urchins collected at monthly intervals throughout one annual reproductive cycle were treated with tritiated thymidine to determine which gonadal cells (both germinal and non-germinal) were synthesizing DNA at each monthly sample. At five different times during the annual cycle, long-term experiments were initiated to time the latter part of gametogenesis.

MATERIALS AND METHODS

All specimens of *Strongylocentrotus purpuratus* used were collected at low tide from tide pools near Yankee Point, California. Most of the urchins collected weighed between 20 g. and 40 g. and had apparently gone through at least one previous annual reproductive cycle. Samples of 12 urchins were collected at approximately monthly intervals from March, 1963 to February, 1964. On the day of collection, the gonads of the sampled urchins were exposed to tritiated

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thymidine for one hour *in vivo* or *in vitro*. To label gonads *in vivo*, each urchin was injected intracoelomically *via* the peristomial membrane with 0.5 μc. of tritiated thymidine per gram of fresh weight of urchin. Before injection, the aqueous solution of tritiated thymidine obtained from New England Nuclear Corp., Boston (with a specific activity of 2000 mc. per m*M*) was diluted with four parts of sea water. The injected urchin was returned to an aquarium for one hour. Then, one of the five gonads was dissected from the urchin and fixed in sea water-Bouin's fluid. It did not matter which of the five gonads was chosen, since preliminary experiments showed that all acini of all gonads in any individual urchin were at the same stage of development. From the wet weight of the other four gonads, the gonad index was calculated by the following formula: gonad index $= 1.25 \times \frac{\text{weight of four gonads} \times 100}{\text{weight of urchin}}$. The average gonad index for each monthly urchin sample for March, 1963 to February, 1964 is given in Figure 1.

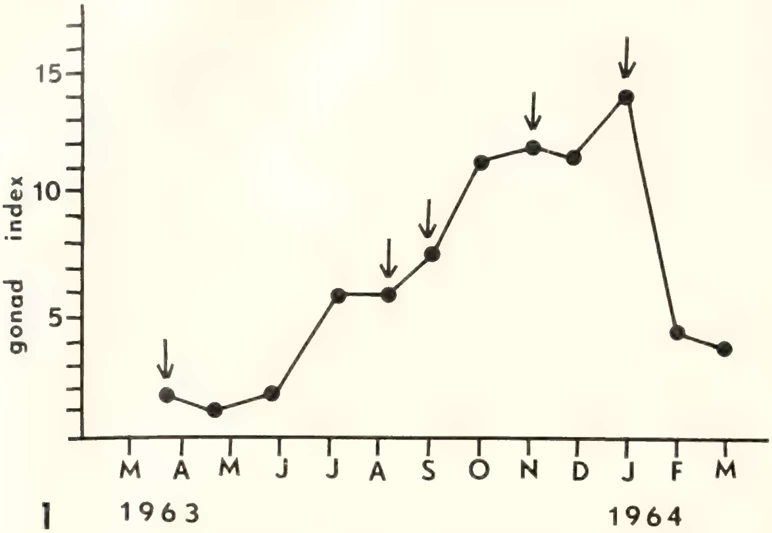


FIGURE 1. The average gonad indices of urchins sampled at approximately monthly intervals during the 1963–1964 annual reproductive cycle. Gonad indices of male and female urchins were considered together for calculation of each point. Long-term experiments were initiated at each of the five points indicated with arrows.

To label gonads *in vitro*, one gonad was removed from each urchin and placed at once in 5 ml. of 14° C. sea water containing 5 μc. of tritiated thymidine. One hour later, the gonad was fixed in sea water-Bouin's fluid. There was no detectable difference in the number or distribution of radioactive nuclei between the *in vivo*- and the *in vitro*-labeled gonads. *In vitro*-labeling used less tritiated thymidine and resulted in more uniform distribution of tritiated thymidine to the gonadal cells than did *in vivo*-labeling, but at the height of the reproductive season, *in vitro*-labeling resulted in considerable loss of mature gametes from the dissected gonads.

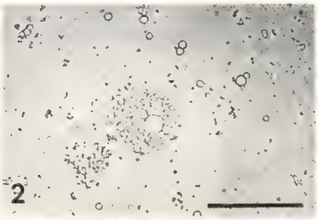
After fixation, the labeled gonads were embedded in paraffin and sectioned at 7 μ and 2 μ. The sections were covered with Kodak AR-10 stripping film; exposure was for 4, 11 or 25 weeks. The autoradiograms were stained through the emulsion with nuclear fast red (Montruil-Langlois, 1962) or with hematoxylin and eosin. Gonad sections adjacent to those prepared as autoradiograms were always stained with hematoxylin and eosin. In each monthly sample, the number of male and female urchins was approximately equal. The gonads from each month's sample usually showed a fairly wide range of developmental stages. Therefore, after the histology for the whole annual cycle had been studied, each month's gonads were rated as typical, retarded, or advanced. Only typical gonads were used in the description of each monthly sample.

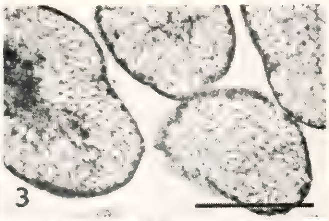
At five different times during the 1963–1964 annual reproductive cycle (indicated by arrows in Figure 1), urchins were collected, injected with 0.5 μc. of tritiated thymidine per gram, and kept in 150-gallon running sea water tanks in the laboratory until sacrificed. In the interval between injection and sacrifice, the urchins were fed as much of the brown alga, *Macrocystis,* as they would eat. At sacrifice, gonad indices were calculated and autoradiograms of labeled gonads were prepared. Of 63 urchins collected and injected on March 22, 1963, 13 were killed after 11 days, 10 after 25 days, 10 after 41 days, 10 after 73 days, 10 after 119 days and 10 after 193 days. Of 19 urchins collected and injected on August 8, 1963, four were killed after 3 days, four after 6 days, four after 9 days, four after 12 days and three after 20 days. Of 31 urchins collected and injected on September 3, 1963, five were killed after 3 days, five after 6 days, five after 12 days, five after 20 days, five after 40 days and six after 100 days. Of 17 urchins collected and injected on November 3, 1963, six were killed after 6 days, six after 20 days and five after 125 days. Of 19 urchins collected and injected on January 1, 1964, six were killed after 6 days, six after 20 days and seven after 65 days.

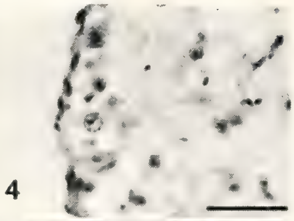
In addition to the 20-g. and 40-g. adult urchins studied, one group of ten very small urchins, ranging from 0.3 g. to 3.2 g., was collected on June 15, 1963. The gonads of these urchins were too small to be used in calculating a gonad index and too small to dissect from the urchin. Therefore, a cut was made around the ambitus of each urchin to obtain the aboral half of the test with the gonads attached. The gonads were labeled *in situ* for one hour in 5 ml. of 14° C. sea water containing 5 μc. of tritiated thymidine. Fixation in sea water-Bouin's fluid decalcified the tests and permitted sectioning for autoradiography of the entire aboral half of the urchin, including the gonads.

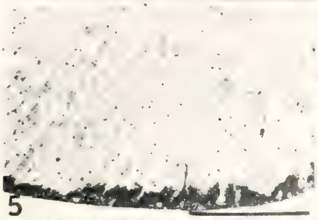
RESULTS

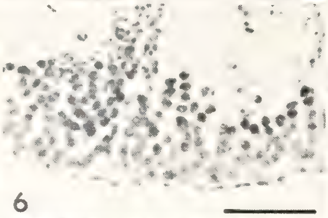
A histological section through an acinus of a sea urchin ovary or testis reveals an outer visceral peritoneum, a middle connective tissue-muscle layer and a lining germinal epithelium. The visceral peritoneum and connective tissue-muscle layer of the gonads of *Arbacia punctulata* were described in detail by Wilson in 1940. In *S. purpuratus,* as in *Arbacia,* the connective tissue-muscle layer is easiest to study in the months after spawning since it becomes stretched very thin and is difficult to see during the ripe season. In the connective tissue-muscle layer of spawned-out gonads, occasional 5 μ × 1 μ nuclei of fibroblasts were labeled by a one-hour exposure to tritiated thymidine. No labeled nuclei of the smooth muscles of this layer were ever detected. Throughout the year, some of the nuclei of the visceral peritoneum of the gonads become labeled by a one-hour exposure to

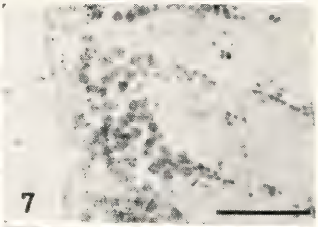


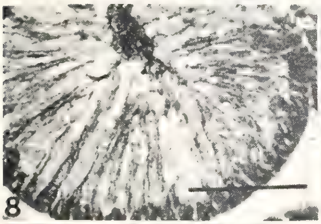


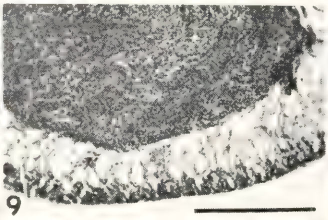


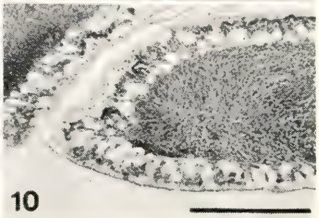


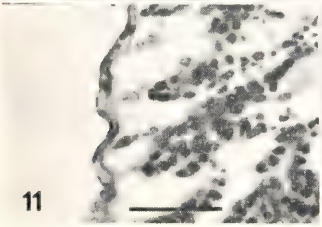


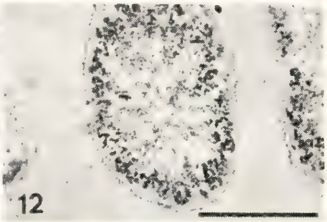












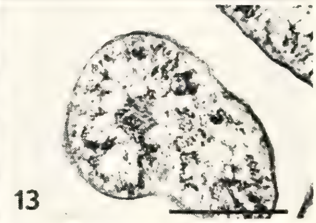


FIGURE 2. A living, globulated nutritive phagocyte in a squash preparation observed by bright-field microscopy. The single vacuole and cytoplasmic globules of reserve are conspicuous. The scale line is 50 μ.

FIGURE 3. A cross-section of acini of a testis from an urchin collected on May 26, 1963. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 4. The periphery of one of the testicular acini of Figure 3. The large nucleus near the base of the germinal epithelium belongs to a primitive spermatogonium, and the smaller nuclei belong to deglobulated nutritive phagocytes. Several relict spermatozoa are also present. The scale line is 20 μ. Hematoxylin and eosin.

FIGURE 5. Part of an acinus of a testis from an urchin collected August 8, 1963. The center of the acinus is at the top of the figure. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 6. The periphery of the testicular acinus of Figure 5. Several trails of spermatids at the top of the figure are proceeding, between the nutritive phagocytes, toward the center of the acinus. Darkly stained spermatocyte nuclei occur along the top border of the basal band of germ cells. At the base of the germinal epithelium are several larger nuclei of primitive spermatogonia. Intermediate in structure and position, between the primitive spermatogonia and spermatocytes, are maturing spermatogonia. The scale line is 25 μ. Hematoxylin and eosin.

FIGURE 7. An autoradiogram of the periphery of the testicular acinus of Figure 5. The base of the germinal layer is toward the left. Some primitive spermatogonia, maturing spermatogonia and spermatocytes are labeled after exposure to tritiated thymidine for one hour. The scale line is 25 μ. Nuclear fast red.

tritiated thymidine. The percentage of labeled peritoneal nuclei is highest in late summer and autumn, when the gonads are increasing in volume. Autoradiograms of gonads from urchins killed several days to several months after injection often show groups of two or more adjacent labeled peritoneal nuclei; each group is probably the progeny of a single initially-labeled cell. These findings support the suggestion of Wilson (1940, p. 472) that "the epithelium accommodates itself to the increasing mass of the gonad by cell division as well as by stretching."

Some workers, such as Caullery (1925), have used the term *germinal epithelium* to refer only to the germinal cells of urchin gonads; there are certain times of year when the germinal cells are widely scattered and not at all organized into an epithelium. This difficulty was avoided in the present investigation by using the term *germinal layer* to include non-germinal as well as germinal cells. At some times of year, the lumen in the center of each acinus may disappear due to encroachment of the non-germinal cells of the germinal layer, but the lumen always reappears as gametes begin to accumulate.

The nutritive phagocytes. The non-germinal cells of the echinoid germinal layer have been described by a number of investigators and have been given several names (see Holland, 1964). The most appropriate name for such cells in *S. purpuratus* is *nutritive phagocyte*. Nutritive phagocytes in the testes and ovaries are similar (aside from differences in the objects they ingest by phagocytosis) and may be discussed together. As Caullery (1925) pointed out, the nutritive phagocytes of echinoid gonads alternate between two morphological phases —globulated and deglobulated. In the globulated phase (July to December of 1963 in the specimens of *S. purpuratus* collected during the present investigation), each nutritive phagocyte is typically a spherical (35 μ in diameter) to oval (45 μ × 25 μ) cell containing conspicuous cytoplasmic globules from 1 μ to 12 μ in diameter. A single, large vacuole is often present. Living nutritive phagocytes (Fig. 2) may easily be demonstrated by gently squashing a small piece of gonad in a drop of sea water beneath a coverslip. In gonad sections stained with hematoxylin and eosin, the typical nutritive phagocyte has an oval nucleus 3 μ × 4 μ which contains a single, poorly-defined nucleolus and little chromatin. The cytoplasmic globules survive fixation and stain strongly with eosin. It is probable that all such globules seen in squash preparations (Fig. 2) come from the cytoplasm of cells disrupted by squashing. Staining with hematoxylin and eosin demon-

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FIGURE 8. Part of an acinus of a testis from an urchin collected October 4, 1963. The sperm-filled lumen is at the top. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 9. Part of an acinus of a testis from an urchin collected November 3, 1963. The sperm-filled lumen is at the top. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 10. Parts of two acini of a testis from an urchin collected on January 1, 1964. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 11. The periphery of an acinus of a testis from an urchin collected on January 30, 1964. Several primitive spermatogonia with large nuclei are attaching clumps of maturing spermatogonia and spermatocytes to the base of the germinal epithelium. The scale line is 25 μ. Hematoxylin and eosin.

FIGURE 12. An autoradiogram of a testicular acinus from an urchin collected on January 30, 1964, and exposed to tritiated thymidine for one hour. Many of the maturing spermatogonia and spermatocytes are heavily labeled. The scale line is 150 μ. Nuclear fast red.

FIGURE 13. An acinus of a testis from an urchin collected on February 27, 1964. Clumps of germ cells, detached from the base of the germinal epithelium, are proceeding toward the center of the acinus. The scale line is 150 μ. Hematoxylin and eosin.

strates cells and cellular debris ingested by the nutritive phagocytes; the ingested objects, which cannot be seen in squash preparations, are usually located at the center of an eosinophilic globule. The cell boundaries of the nutritive phagocytes are usually inconspicuous in stained sections of gonad.

In November and December, as the gonad index was approaching its maximum value (1963–1964 annual reproductive cycle), the nutritive phagocytes as seen on the slides begin to change to their alternate morphological state. They shrink to 15 μ in diameter and contain fewer and smaller cytoplasmic globules. Most of the globules shrink to a diameter less than 1 μ or disappear entirely from the testis by December 1, 1963, and from the ovary by January 1, 1964; a month's difference in the time of disappearance of the globules is the only dissimilarity between the nutritive phagocytes of the male and female. No change in nuclear morphology accompanies the loss of globules from the cytoplasm, and the single vacuole is often retained. Deglobulated nutritive phagocytes which retain their vacuoles appear as hollow spheres (usually stuck to one another in clumps) in squash preparations. In stained sections, these vacuolated cells give the germinal epithelium a net-like appearance referred to collectively as the *netzförmigen Syncytium* by Lindhal (1932, p. 382); however, it is likely that they are individual cells and are not organized into a syncytium. In some ripe urchins, especially those that became abnormally ripe due to their failure to spawn in the laboratory, the nutritive phagocytes lose their vacuoles and can only be detected as scattered nuclei in the attenuated germinal layer. Such devacuolated nutritive phagocytes cannot be detected at all in squash preparations. After spawning, the deglobulated nutritive phagocytes remain in their alternate morphological state until the gonad index begins to rise; then cytoplasmic globules reappear.

Autoradiograms of gonads treated for one hour with tritiated thymidine reveal that many of the deglobulated nutritive phagocytes synthesize DNA, presumably in preparation for mitosis. Labeled nuclei of nutritive phagocytes occur at all levels in the germinal layer. Therefore, the production of new nutritive phagocytes is not limited to the base of the germinal layer (as Caullery believed in 1925). Nutritive phagocytes in their deglobulated phase labeled on March 22, 1963 (and their progeny), can still be detected when they have reverted to the globulated state several months after injection. As the gonad index begins to rise and the cytoplasm of each nutritive phagocyte as seen on the slides begins to fill with globules, the number of nutritive phagocytes synthesizing DNA declines. At the height of the reproductive season, no labeled nutritive phagocytes are detected after a one-hour exposure to tritiated thymidine. Thus, the nutritive phagocytes apparently originate from pre-existing nutritive phagocytes dividing by mitosis in the germinal layer of unripe gonads. This conclusion does not agree with earlier schemes deriving the nutritive phagocytes from cells of the visceral peritoneum covering the gonad (Tennent *et al.,* 1931; Miller and Smith, 1931), or from coelomocytes (Liebman, 1950).

Nutritive phagocytes were found in the gonads of all the immature specimens of *S. purpuratus* investigated. In most of these immature gonads, the recognizable nutritive phagocytes observed are vacuolated, but lack eosinophilic globules. Nutritive phagocytes in the gonad of the largest immature urchin (3.2 g) investigated contained a few eosinophilic globules. Exposure of the immature gonads to tritiated thymidine for one hour labeled many of the nutritive phagocytes. At present, it is not known whether nutritive phagocytes can be found in the gonads of specimens of *S. purpuratus* weighing less than 0.3 g. From the descriptions of Hamann (1888) and MacBride (1903), nutritive phagocytes are apparently not found at the gonadal tube stage and earlier in the life history of the sea urchin. This raises the question of the ultimate source of the nutritive phagocytes. Cuénot in 1892 supplied a possible answer: that germinal cells and nutritive phagocytes both arise from a common primitive cell type very early in the life history of the urchin. These primitive cells may possibly be the *Urkeimzellen* of Hamann (1888) and the "large cells" of MacBride (1903).

The testis. It is most convenient to begin a description of the annual cycle of the urchin testis with the urchins sampled on April 21 and May 26, 1963 (Fig. 3). At this stage of the annual reproductive cycle the germinal layer consists chiefly of vacuolated, deglobulated nutritive phagocytes and the acinar lumens may or may not contain masses of relict spermatozoa, that is, spermatozoa left over from a previous spermatogenesis. Such spermatozoa are usually scattered at all levels in the germinal layer; it is probable that most of them lie within the cytoplasm of the nutritive phagocytes, although this cannot be rigorously demonstrated by light microscopy (Figs. 3 and 4). A few scattered spermatogonia lie at the base of the germinal layer. These spermatogonia, which are usually delimited by a distinct cell membrane, have a small amount of clear or slightly basophilic cytoplasm. Their nuclei are at most about 5 μ in diameter and usually have one or two nucleoli and little chromatin (Fig. 4). In autoradiograms of typical testes from the April 21 and May 26 urchin samples, some of the spermatogonia are labeled by exposure to tritiated thymidine for one hour. DNA synthesis by the spermatogonia is presumably followed by mitotic division. In places at the base of the germinal layer where the spermatogonia have divided several times, they are grouped in cell nests. Such spermatogonia will be referred to hereafter as *primitive spermatogonia.*

The germinal layer of a typical testis from the urchins sampled July 8, 1964, consists mostly of nutritive phagocytes, now containing eosinophilic globules as well as relict spermatozoa; masses of such spermatozoa are still found in a few of the acinar lumens. The germ cell nests at the base of the germinal epithelium contain primitive spermatogonia and also a second type of cell which has a basophilic cytoplasm and a nucleus 3 μ to 4 μ in diameter; the nucleus often lacks a nucleolus and contains more chromatin than the nucleus of a primitive spermatogonium. It is likely that cells of this type are maturing spermatogonia (which may be compared to the intermediate and type B spermatogonia of mammals). A one-hour exposure to tritiated thymidine labels many of the primitive and maturing spermatogonia.

In the typical testes sampled on August 8, 1963, the individual nests of spermatogonia have become confluent, creating a continuous band of germ cells at the base of the germinal layer (Fig. 5). The cells of this band are by now so crowded that individual cell boundaries can rarely be seen even in 2-μ sections. Therefore, it is necessary to describe cell types by nuclear morphology alone. In general, the primitive spermatognia are the most basal cells in the band, and the spermatocytes in the band are closest to the lumen (Fig. 6). The spermatocyte nuclei are 3 μ to 4 μ in diameter, and some are filled with a thread-like chromatin which stains strongly. Other spermatocyte nuclei contain numerous, stubby chromosomes, which give them a lumpy appearance. There is no reliable criterion for setting apart primary and secondary spermatocytes in *S. purpuratus,* although Nishikawa (1961) reported that secondary spermatocytes contain thinner chromosomes than primary spermatocytes in two Japanese sea urchins. Also in the band of germ cells are numerous cells intermediate in nuclear morphology as well as position between the spermatocytes and basal primitive spermatogonia. Since mitotic figures are often found among these intermediate cells, it is likely that they constitute a population of maturing spermatogonia rather than early primary spermatocytes. As these maturing spermatogonia intergrade structurally with both the primitive spermatogonia and the spermatocytes, it is impossible to establish precise boundaries between these cell types.

In the typical testes sampled in August, a few trails of spermatids arise from the spermatocytes and extend, in the spaces between adjacent nutritive phagocytes, a short distance toward the center of the acinus (Figs. 5 and 6). The more basal, younger spermatids have darkly staining, round nuclei about 2 μ in diameter, while the more mature spermatids have more elongated nuclei, which resemble blunt spermatozoa; spermatid cytoplasm is not demonstrable. A few newly-formed spermatozoa arise from the spermatids in the spaces between adjacent nutritive phagocytes. These spermatozoa have pointed conical heads, 2.5 μ long and 1.2 μ in maximum diameter, and are morphologically indistinguishable from relict spermatozoa in the acinar lumen or in the nutritive phagocytes.

Many primitive spermatogonia, maturing spermatogonia and spermatocytes are labeled by a one-hour exposure to tritiated thymidine (Fig. 7). The labeled primitive and maturing spermatogonia are synthesizing DNA in preparation for mitotic division. It is likely that mitotic divisions of primitive spermatogonia produce both primitive spermatogonia and maturing spermatogonia, while all maturing spermatogonia go through a limited number of mitotic divisions and then differentiate into primary spermatocytes. Thus, the primitive spermatogonia probably constitute the self-sustaining, progenitor compartment and the maturing spermatogonia probably constitute an amplification compartment of germ cell production in the testes. The labeled spermatocytes are presumably pre-leptotene primary spermatocytes (comparable to the resting primary spermatocytes of mammals) synthesizing DNA in preparation for meiotic divisions.

In testes from urchins injected with tritiated thymidine on August 8 and killed three days later, the only labeled germinal cells are primitive spermatogonia, maturing spermatogonia, and spermatocytes. Six days after August 8 injection, the most advanced labeled cells are the younger spermatids. Twelve and 20 days after the August 8 injection, the most advanced labeled cells are spermatozoa. Therefore, the minimum time required in August for a pre-leptotene primary spermatocyte to become a spermatid is between three and six days. This time is probably closer to six days than three days, since only the younger spermatids are labeled at six days. The minimum time required in August for a pre-leptotene primary spermatocyte to become a mature sperm is more than six and less than 12 days. This time is probably somewhat less than 12 days (probably about 10 days), judging from the large number of labeled spermatozoa present 12 days after injection. In several testes from the August sample, the cytoplasm of the nutritive phagocytes contains many ingested spermatozoa embedded, sometimes singly and sometimes in clumps, within the eosinophilic globules. Twelve and 20 days after injection of tritiated thymidine, some of these ingested spermatozoa are labeled. Therefore, the nutritive phagocytes ingest not only relict spermatozoa, but also some newly-formed spermatozoa, and sometimes spermatids and spermatocytes.

A typical testis from the sample taken September 3, 1963, resembles a typical testis from the previous month, except the basal band of germ cells is thicker. As in the previous month, many primary spermatogonia, maturing spermatogonia, and spermatocytes are labeled by a one-hour exposure to tritiated thymidine. In testes from urchins injected with tritiated thymidine on September 3 and killed three days later, only primary spermatogonia, maturing spermatogonia, and spermatocytes are labeled. Six days after injection, the label has reached the younger, spherical spermatids. In testes from urchins injected on September 3 and kept in the laboratory for 20 days, continuous trails of spermatids and spermatozoa lead, in the spaces between the nutritive phagocytes, from the basal band of germ cells to the lumen, which contains a small mass of spermatozoa (see Fig. 8). Thus, by this time, at least some of the spermatozoa in the lumen are newly-produced and not from a past reproductive cycle. This conclusion, based on anatomical evidence, is borne out by autoradiograms showing labeled spermatozoa in the central sperm mass 20 days after injection. In the same testes, some nutritive phagocytes contain ingested labeled spermatozoa. In testes sampled 40 days after the September 3 injection, the lumen contained labeled spermatozoa, while some ingested clumps of labeled spermatozoa are still seen in the nutritive phagocytes. In testes sampled 100 days after the September 3 injection, labeled spermatozoa are still present in the lumen. After 100 days, no labeled spermatozoa are found in the cytoplasm of the nutritive phagocytes, but labeled amorphous basophilic granules are sometimes present; presumably these labeled granules are the remains of partially digested, labeled spermatozoa.

In typical testes from the samples of October 4 and November 3, 1963, the acinar lumen contains a mass of newly-produced spermatozoa (Figs. 8 and 9). A broad band of primitive spermatogonia, maturing spermatogonia and spermatocytes occupies the basal part of the germinal layer. Streams of spermatids and spermatozoa are proceeding between adjacent nutritive phagocytes toward the lumen. A one-hour exposure to tritiated thymidine labels many primitive spermatogonia, maturing spermatogonia, and spermatocytes. In testes from urchins injected with tritiated thymidine on November 3 and killed six days later, the label has reached the younger, spherical spermatids. Twenty days after the injection of November 3, large numbers of labeled spermatozoa are found in the central sperm mass. Twenty days after injection, no labeled spermatozoa can be detected in the cytoplasm of the nutritive phagocytes. Apparently the phagocytosis of spermatozoa by the nutritive phagocytes of the testes ceases between September and November. The cessation of phagocytosis at approximately the same time that spermatozoa begin to accumulate in the lumen suggests that the nutritive phagocytes may play an important part in regulating the rate of accumulation of spermatozoa in the lumen. Testes from urchins injected on November 3, 1963, still contain labeled spermatozoa on March 7, 1964 (135 days later). Apparently these urchins, which had an average gonad index of 17.1 at sacrifice, failed to spawn in the laboratory during the first part of January, 1964, when the urchins in the field spawned.

In typical testes from urchins sampled on December 1, 1963, and January 1, 1964, the acinar lumen is distended with a great mass of spermatozoa. The germinal layer is markedly thinner than in earlier months, partly due to shrinkage of the nutritive phagocytes (which have lost most or all of their eosinophilic globules), and partly due to a thinning of the basal band of germ cells. Streams of spermatids and spermatozoa are still proceeding between nutritive phagocytes toward the lumen. In the testes of some individuals (Fig. 10), deglobulated nutritive phagocytes occur among the spermatocytes, maturing spermatogonia, and primitive spermatogonia. Some of these nutritive phagocytes appear to detach parts of the basal band of germ cells from the basement membrane which separates the germinal epithelium from the connective tissue-muscle layer. As in previous months, primitive spermatogonia, maturing spermatogonia, and spermatocytes are labeled by exposure to tritiated thymidine for one hour. In testes from urchins injected with tritiated thymidine on January 1, 1964, and killed six days later, the label has reached the spermatids. Twenty days after injection, there are numerous labeled spermatozoa in the acinar lumen. No testes were available from the urchins injected on January 1, 1964, and sacrificed on March 7, 1964, since all were females.

In a typical testis from urchins sampled on January 30, 1964, several weeks after spawning (A. L. Lawrence, personal communication), the acinar lumen contains only a small number of spermatozoa. The germinal layer still consists of deglobulated nutritive phagocytes and a basal region of germ cells. Many nutritive phagocytes occur among the basal germ cells and divide them into separate clusters of cells, which remain attached by one or two cells to the basement membrane. In many clusters, the most basal cells are primitive spermatogonia (Fig. 11). It is most likely that these cells are the very spermatogonia from which will spring the germ cells of the next annual reproductive cycle. Treatment of such a testis for one hour with tritiated thymidine labels many of the maturing spermatogonia and spermatocytes in the cell clusters (Fig. 12). The primitive spermatogonia at the base of the cell clusters are labeled only rarely.

Typical testes from urchins sampled on February 27, 1964, and on March 22, 1963, have deglobulated nutritive phagocytes and clumps of germ cells at all levels in the germinal layer (Fig. 13). Most of these clumps of maturing spermatogonia and spermatocytes have become detached from the base of the germinal layer and appear to be moving toward the acinar lumen. The lumen contains spermatozoa, often mixed with clumps of spermatids and spermatocytes. A few scattered primitive spermatogonia remain near the base of the germinal layer. Exposure of such a testis to tritiated thymidine for one hour labels many of the maturing spermatogonia and spermatocytes, even as they are progressing toward the lumen in disorganized clumps few of the primitive spermatogonia are labeled. In testes from urchins injected with tritiated thymidine on March 22, 1963, and killed 11 days later, the acinar lumen contains some labeled spermatozoa. Therefore, spermatozoa are produced after spawning has occurred, and the term *relict* *spermatozoa* should not necessarily connote spermatozoa produced before spawning. Some labeled spermatozoa were detected in the testes of urchins injected on March 22, 1963, and killed at increasing time intervals (up to 193 days) thereafter.

The ovary. During the 1963–1964 annual reproductive cycle, germ cells were synthesizing DNA in typical ovaries sampled from March through October. These germ cells, which include oogonia and primary oocytes, occur in small groups or nests near the base of the germinal layer. In most ovaries, these cell nests are inconspicuous, and several acinar cross-sections must usually be searched before a nest is located. The oogonia have scanty, clear cytoplasm and a nucleus 5 μ in diameter; the oogonial nucleus contains one or two indistinct nucleoli and little chromatin. The pre-leptotene primary oocytes have a thin shell of clear cytoplasm and a nucleus 4 μ to 5 μ in diameter; a nucleolus is often present and the chromatin is organized into fine threads. The cell nests also contain primary oocytes from the leptotene through the diplotene stages of the first meiotic prophase; the chromatin of these oocytes is organized into a spireme. After the primary oocytes complete the diplotene stage, they enter the interphase-like dictyotene stage. Tennent and Ito (1941) referred to the dictyotene stage as the *diffusion stage* plus the *resting nucleus stage*. The primary oocytes remain in the dictyotene stage until they are fully grown and ready to undergo maturation divisions; only then does the first meiotic prophase terminate. The smallest primary oocytes in the dictyotene stage are scattered at the base of the germinal epithelium.

Exposure to tritiated thymidine for one hour labels some of the oogonia and pre-leptotene primary oocytes in each cell nest[3] (Fig. 14). As early as three days after injection of tritiated thymidine, some of the primary oocytes in the spireme stage have become labeled. In none of the long-term experiments can the label be traced into growing primary oocytes, secondary oocytes and mature ova. Therefore, it may well be that the label remains in the smallest primary oocytes in the dictyotene stage until the following annual reproductive cycle when the labeled primary oocytes grow and undergo maturation divisions. Such an extended dictyotene stage in the human primary oocyte has been reported to last many years (Ohno *et al.*, 1962; Baker, 1963), while a dictyotene stage lasting many months apparently occurs in female mice (Rudkin and Griech, 1962) and in female chicks (Hugres, 1963).

It is convenient to begin a description of the annual cycle of the ovary with specimens of *S. purpuratus* sampled on May 26, 1963. A typical ovary for this stage in the annual reproductive cycle is shown in Figure 15. Most of the acinar lumens contain a few ova from a preceding reproductive cycle (relict ova), some but not all of which are being attacked and digested by deglobulated vacuolated nutritive phagocytes. The germinal layer, in addition to the nutritive phagocytes and germ cell nests already described, has as its base a number of small primary oocytes, apparently produced during the preceding annual reproductive cycle. These elliptical oocytes have a weakly basophilic, fibrous cytoplasm and a spherical nucleus containing a strongly basophilic, spherical nucleolus. The largest of these primary oocytes is 15 μ × 12 μ and has a nucleus 9 μ in diameter containing a nucleolus 3 μ in diameter. In typical ovaries (Fig. 16) from urchins collected July 8 and August 7, 1963, the nutritive phagocytes have eosinophilic

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[3] These results are in general agreement with the earlier findings of Nigon and Gillot (1963), who incubated fragments of *Arbacia lixula* ovary in the spring for one hour in sea water containing tritiated thymidine and found a few labeled cells which they interpreted as "jeunes ovocytes" (p. 249). Nigon and Gillot did not attempt to follow the fate of the labeled cells in long-term experiments *in vivo*.

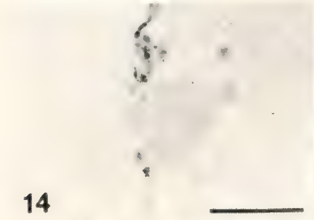
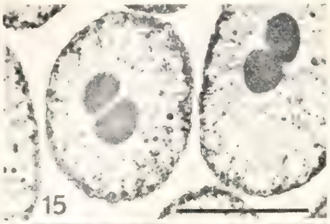
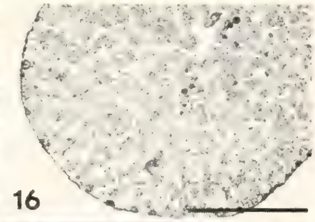
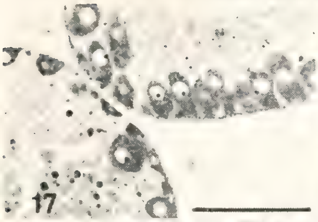
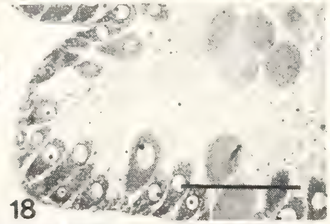
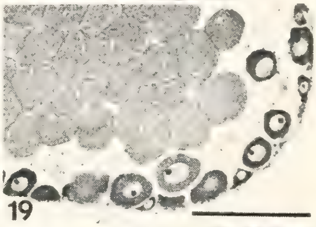
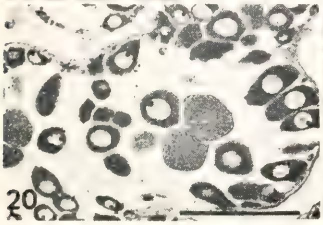
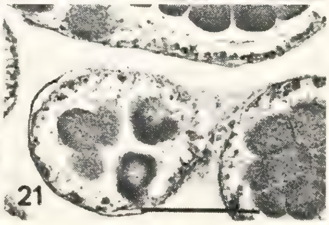
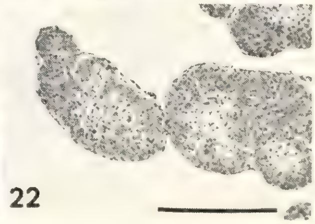

FIGURE 14. An autoradiogram prepared from an ovary of an urchin collected September 3, 1963. Above the large, growing primary oocyte, there is a nest of oogonia and pre-leptotene primary oocytes labeled after exposure to tritiated thymidine for one hour. One of the cells of the visceral peritoneum is also labeled (below the growing primary oocyte). The scale line is 25 μ. Nuclear fast red.

FIGURE 15. Several acini of an ovary from an urchin collected May 26, 1963. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 16. Part of an acinus of an ovary from an urchin collected July 8, 1964. The center of the acinus is at the top. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 17. Parts of two acini of an ovary from an urchin collected October 4, 1963. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 18. Part of an acinus of an ovary from an urchin collected on November 2, 1963. The center of the acinus is at the top right. A primary oocyte of maximum size and an oocyte with a maturation spindle are proceeding, in the spaces between adjacent nutritive phagocytes, toward the acinar lumen. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 19. Part of an acinus of an ovary from an urchin collected on January 1, 1964. The center of the acinus is at the upper right. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 20. An acinus of an ovary from an urchin collected on January 30, 1964. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 21. Several acini of an ovary from an urchin collected on March 22, 1963. Many large oocytes and ova are being digested by deglobulated nutritive phagocytes. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 22. Several acini of an ovary from a very small (0.3 g.), immature female specimen of *S. purpuratus*. A number of 15 μ × 12 μ primary oocytes may be seen. The scale line is 150 μ. Hematoxylin and eosin.

globules in their cytoplasm, and relict ova are only rarely seen in the acinar lumens. In all other respects, these ovaries resemble the typical ovary from the sample of May 26, 1963.

In typical ovaries from urchins collected on September 3, 1963, germ cell nests are still present, but many of the primary oocytes at the base of the germinal epithelium are larger and have a more basophilic cytoplasm than in the previous months. The largest of these oocytes is 30 μ × 25 μ; each has a nucleus 15 μ in diameter containing a nucleolus 5 μ in diameter. In typical ovaries sampled on October 4, 1963, the growing primary oocytes with strongly basophilic cytoplasm continue to lie at the base of the germinal epithelium (Fig. 17). The largest of these oocytes is 45 μ × 30 μ and has an elliptical nucleus 22 μ × 18 μ containing a nucleolus 6 μ in diameter. October is the last month in which the germ cell nests occur at the base of the ovarian germinal epithelium.

Typical ovaries sampled November 2, 1963, contain many primary oocytes of maximum size, some at the base of the germinal epithelium and others proceeding between the nutritive phagocytes toward the lumen (Fig. 18). A primary oocyte of maximum size (which many embryologists term an "immature egg" or an "egg in the germinal vesicle stage") is usually elliptical (65 μ × 45 μ) and has weakly basophilic cytoplasm; the nucleus (or germinal vesicle) is 28 μ × 22 μ and contains a nucleolus 8 μ in diameter. Some of the oocytes of maximum size (secondary as well as primary) contain maturation spindles (Fig. 18) and are giving off polar bodies. Maturation can occur at any level in the germinal epithelium or in acinar lumen. The acinar lumen contains a few newly-produced ova, which are 60 μ in diameter and each contains a nucleus 9 μ in diameter. Since the diameter of a living ovum of *S. purpuratus* is about 80 μ, it is evident that fixation and embedding causes considerable shrinkage. At the base of the germinal epithelium of ovaries sampled in November, there are growing primary oocytes of assorted sizes and also a number of small cells resembling the oogonia described previously. It is not clear whether these cells are oogonia, very small primary oocytes in the dictyotene stage, or both; they are not labeled by a one-hour exposure to tritiated thymidine. Typical ovaries from the urchins sampled on December 1, 1963, resemble the typical ovaries of the previous month, but have far more ova in the acinar lumens.

The typical ovaries sampled on January 1, 1964, contain more ripe ova than ovaries sampled December 1, 1963, and the nutritive phagocytes have become deglobulated (Fig. 19). In a typical ovary from urchins sampled on January 30, 1964, several weeks after spawning, the acinar lumen contains only a small number of ova, while the germinal layer is much the same as it was on January 1, 1964. Some primary oocytes of maximum size and some oocytes in the process of maturation are still present (Fig. 20). It is, therefore, very likely that large primary oocytes continue to mature into ova after spawning has occurred. The large oocytes and the mature ova show no signs of being digested by the nutritive phagocytes.

The ovaries sampled on February 27, 1964, unlike ovaries of the preceding months, contain no medium-sized primary oocytes. At the base of the germinal epithelium are only small primary oocytes, the largest of which is 20 μ × 15 μ, each with a nucleus 12 μ in diameter containing a nucleolus 4 μ in diameter. The acinar lumens contain some ova, a number of primary oocytes of maximum size, and a few oocytes in the process of maturation. A few of these ova and oocytes are being attacked and digested by the nutritive phagocytes. The disappearance of the medium-sized oocytes may be due to the completion of growth and maturation by all oocytes larger than some critical size (which is apparently 20 μ × 15 μ), while all oocytes smaller than this remain, without growing, at the base of the germinal epithelium.

Spawning in 1964 occurred as in 1963, during the month of January (A. L. Lawrence, personal communication). Therefore, gonads sampled in late March of 1964 and in late March of 1963 should be at a similar stage of development. In ovaries sampled March 22, 1963, many of the ova and oocytes of maximum size are being attacked and destroyed by the nutritive phagocytes (Fig. 21). Where the attacking nutritive phagocytes come into contact with an ovum or oocyte, the boundary of the attacked cell becomes indistinct and its cytoplasm appears to be eroded away. At the base of the germinal layer, the germ cell nests are again present. These nests are probably reconstituted by mitosis of reserve oogonia carried over from the preceding fall. As in the previous month, the largest primary oocytes are still 20 μ × 15 μ and each has a nucleus 12 μ in diameter containing a nucleolus 4 μ in diameter.

A typical ovary from urchins sampled on April 21, 1963, has only a few ova remaining from the preceding oogenesis and no primary oocytes of maximum size left in the acinar lumens. The nutritive phagocytes continue to attack some of these ova and also appear to be destroying some of the larger (20 μ × 15 μ) primary oocytes at the base of the germinal layer. By May 26, 1963, when the sample which began this description of the annual ovarian cycle was taken, the largest primary oocytes remaining at the base of the germinal layer are 15 μ × 12 μ; each has a nucleus 9 μ in diameter containing a nucleolus 3 μ in diameter.

Immature gonads. In all specimens of *S. purpuratus* weighing less than 3.0 g. (16 mm. in test diameter), the gonads remain immature throughout the year, even at the height of the reproductive season for the majority of the population. Although Fuji (1960) reported that the gonads of small specimens of *Strongylocentrotus intermedius* are neuter, the sex of small specimens of *S. purpuratus* can be determined by microscopic investigation of their gonads. The germ cells of immature ovaries include oogonia, pre-leptotene primary oocytes, primary oocytes in the spireme stages of the first meiotic prophase and primary oocytes in the dictyotene stage. The largest of these dictyotene primary oocytes is 15 μ × 12 μ and has a nucleus 10 μ in diameter containing a nucleolus 3 μ in diameter (Fig. 22). Exposure of the immature ovaries to tritiated thymidine for one hour labels many of the oogonia and pre-leptotene primary oocytes. The primary oocytes which were produced on June 15, 1963, were presumably those which would grow and mature during the 1964–1965 reproductive cycle. The only germ cells in the testes of immature urchins are apparently primitive spermatogonia. A one-hour exposure to tritiated thymidine labels some of the primitive spermatogonia in these testes.

DISCUSSION

The present investigation has elucidated the time course of the later events of spermatogenesis in *S. purpuratus.* The interval from primary spermatocyte DNA synthesis to early spermatid is about six days. It is probable that each primary spermatocyte spends most of this six-day period in an extended first meiotic prophase, and then divides into two secondary spermatocytes, each of which quickly divides into two early spermatids. Since the first labeled spermatozoa appear tbout 10 days after labeling, approximately four days are required for a spermatid to differentiate into a mature spermatozoon (the process of spermiogenesis). The duration of the later phases of spermatogenesis in the sea urchin is comparable with those in the fruit fly, where the interval from primary spermatocyte DNA synthesis to early spermatid is five days and where spermiogenesis takes three days (Chandley and Bateman, 1962). These intervals in both the sea urchin and the fruit fly are considerably shorter than in the laboratory rat. In the rat, the interval from primary spermatocyte DNA synthesis to early spermatid is 18 days, while the interval from early spermatid to ripe spermatozoan is approximately 17 days (Clermont *et al.,* 1959).

In *S. purpuratus,* the time-course for the later events of spermatogenesis was the same for the production of spermatozoa in August, September, November, January, and March of the 1963–64 annual reproductive cycle. This uniformity in the duration of the cellular transitions between primary spermatocyte DNA synthesis and the appearance of mature spermatozoa means that this part of spermatogenesis can exert no control over the rate of sperm production. Therefore, the events which control the rate of sperm production must occur prior to the termination of DNA synthesis by the primary spermatocytes. The nature of these controlling events was not elucidated in the present investigation. Several possible schemes may be proposed to explain the seasonal fluctuations in the rate of sperm production. In the simplest of these, sperm production could be controlled by seasonal fluctuations in the duration of the growth-duplication cycle of the primitive spermatogonia. For example, in the spring the average duration of the growth-duplication cycle could be several weeks or months, but during the late summer and fall it may shorten to about one day.

In *S. purpuratus,* the rate of sperm production does not necessarily equal the rate of sperm accumulation in the lumen of the testis. Early in the ripe season, newly produced spermatozoa are often ingested by the nutritive phagocytes, and never reach the lumen. Therefore, the rate of sperm accumulation in the lumen is determined both by the rate of sperm production and by the phagocytic activity of the nutritive phagocytes.

In the ovary of *S. purpuratus,* it is probable that the rate of increase in size of the primary oocytes (and not the rate of their production the year before) determines the rate of production of ripe ova. The possibility that the nutritive phagocytes of the ovary exert a partial control over production of ova was neither proved nor disproved in the present investigation. Since, in any given year, there is no significant difference in the gonad index curve of male and female specimens of *S. purpuratus* (Bennett and Giese, 1955), it is possible that the same factor or factors which influence spermatogonial proliferation may influence oocyte growth in the female.

For a number of years, the annual reproductive cycle of *S. purpuratus* has been studied by monthly determinations of the gonad index of a periodically-sampled population of urchins (Giese, 1959). The shape of the gonad index curve and the time of spawning are often strikingly different from one year to the next. Spawning during the 1963–1964 annual reproductive cycle studied was in January of 1964. Spawning of the Yankee Point population of *S. purpuratus* has been as late as May (Bennett and Giese, 1955). At present, it is not known whether the time-course of germ cell proliferation and growth in years of early spawning and years of late spawning is different.

The nutritive phagocytes, in addition to the phagocytic activity by which they destroy relict gametes and control the accumulation of spermatozoa, also have a nutritive function in *S. purpuratus.* The cytoplasmic globules probably arise in part from ingested gametes and in part from nutrients translocated to the gonads by the coelomic fluid and hemal system. The cytoplasmic globules probably have a complex composition; a conspicuous component is a neutral mucopolysaccharide-protein complex (Holland, 1964). In the gonads of *S. purpuratus,* the globules of reserve abruptly disappear as the gonads approach maturity nearing the height of the reproductive season. Even as the globules disappear, the gonad index continues its steady rise due to the rapid increase in cell number in the testis and in the cell size in the ovary. The direct phagocytosis of globules from nutritive phagocytes by growing oocytes, reported for *Arbacia punctulata* by Liebman (1950), was never observed in the ovaries of *S. purpuratus.* Therefore, the reserves stored in the nutritive phagocytes are presumably translocated in a soluble form to the germ cells (and possibly, to some extent, to other tissues as well). The globules in the nutritive phagocytes apparently were not utilized until most of the reserves stored in the inner epithelium of all major gut regions had been depleted during October and November of 1963 (A. L. Lawrence and J. M. Lawrence, personal communication). It is probable that a large part of these gut reserves was translocated to the gonads to support germ cell growth and proliferation early in the ripe season.

SUMMARY

1. The DNA-synthesizing cells in the gonads of the purple sea urchin were labeled with tritiated thymidine and detected with autoradiography.

2. Some fibroblasts in the connective tissue-muscle layer and some cells of the visceral peritoneum covering the gonads synthesize DNA.

3. Some of the non-germinal cells (the nutritive phagocytes) of the germinal layer proliferate during the spring and early summer, but proliferation ceases in the late summer as the nutritive phagocytes begin to accumulate cytoplasmic globules of reserve. Nutritive phagocytes labeled with tritiated thymidine in their deglobulated phase in the spring are still labeled up to several months later, after they have acquired cytoplasmic globules.

4. In the testes of male urchins at the beginning of the reproductive season, not only relict spermatozoa, but also some newly-formed spermatozoa are ingested by the nutritive phagocytes. The phagocytosis of newly-formed spermatozoa ceases later in the reproductive season, at approximately the same time that spermatozoa begin to accumulate in the lumen.

5. In the germinal epithelium of the testis, the germ cells which synthesize DNA are the spermatogonia and primary spermatocytes. Primary spermatocytes labeled with tritiated thymidine differentiate into early spermatids in about six days, while spermiogenesis takes approximately four days.

6. Throughout the portion of the annual reproductive cycle when spermatozoa are being produced (August through March), the time course for the later events of spermatogenesis remains constant. Therefore, the events which control the rate of sperm production must occur early in spermatogenesis.

7. In the germinal layer of the ovary, the germ cells which synthesize DNA are the oogonia and the pre-leptotene primary oocytes. During the same annual reproductive cycle, long-term experiments failed to demonstrate the differentiation of pre-leptotene primary oocytes into growing primary oocytes, maturing oocytes or ova. Therefore, it is likely that labeled primary oocytes remain small and inconspicuous until the following annual reproductive cycle when they grow and mature into ova.

8. The sex of very small, immature urchins may be determined by histological examination of their gonads. The ovaries of immature female urchins contain small primary oocytes which are presumably the source of ova developing during the first annual reproductive cycle.

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AN AUTORADIOGRAPHIC INVESTIGATION OF COELOMOCYTE PRODUCTION IN THE PURPLE SEA URCHIN (STRONGYLOCENTROUS PURPURATUS)[1]

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Coelomocyte is a generic term for several cell types which circulate in the fluid of the coelomic cavities and water vascular system of sea urchins. Boolootian and Giese (1958) have described the living coelomocytes from the perivisceral coelom of *Strongylocentrotus purpuratus*. They recognized the following seven cell types: bladder amebocytes, filiform amebocytes, colorless spherule amebocytes, eleocytes, vibratile cells, fusiform corpuscles, and hyaline hemocytes. It is well known that two types of coelomocyte, the eleocytes and colorless spherule amebocytes, not only circulate in the coelomic and water vascular fluid, but also wander freely throughout most tissues and organs of the urchin. Several workers have claimed that bladder and filiform amebocytes may occur in the gut wall (Kindred, 1926; Saint-Hilaire, 1897). What little is known of the functions of echinoid coelomocytes was summarized by Boolootian and Giese (1958, 1959).

Echinoid coelomocyte renewal has long been a subject of speculation and controversy. Several organs and tissues have been named, chiefly on histological evidence, as the site of coelomocyte production in sea urchins. A number of workers believed that the axial organ was the site of coelomocyte production (see Kollmann, 1908, p. 184). Kollmann claimed that, in addition to the cells of the axial organ, some types of circulating coelomocytes divided to produce more coelomocytes. Kollmann explained his inability to find any mitotic activity in the circulating coelomocytes or axial organ cells by implying that coelomocyte production was a discontinuous process, and coelomocytes were not being produced in the urchins at the time he investigated them. Like Kollmann, other workers have remarked on the rarity or complete absence of mitosis in the circulating coelomocytes of urchins. Geddes (1880) reported that he had only once or twice seen circulating coelomocytes divide. Yeager and Tauber (1935) looked in vain for mitotic figures in living coelomocytes from 20 specimens of *Arbacia punctulata*. Schinke (1950) was unable to find a single mitotic figure in living coelomocytes from 100 specimens of *Psammechinus miliaris*. Schinke also failed to detect any mitotic figures in fixed and stained coelomocytes. In the same paper, Schinke reported that the dermal connective tissue cells of the body wall were the source of at least some types of coelomocyte. Finally, the epithelial cells of the peritoneum have been named as the source for some or all coelomocyte types (Geddes, 1880; Sainte-Hilaire, 1897; Liebman, 1950).

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There have been several reports of the differentiation of one type of coelomocyte into another type in sea urchins. The term differentiation is used here to exclude the pre-clot transformation of the bladder amebocytes into filiform amebocytes, which is a short-term physiological change (Boolootian and Giese, 1959). Frenzel (1892) believed that eleocytes differentiated into bladder and filiform amebocytes. Kindred (1926) reported that bladder and filiform amebocytes were transformed into colorless spherule amebocytes, which were subsequently converted to eleocytes. Liebman (1950) held that the vibratile cells, after originating from the peritoneum, transformed into bladder and filiform amebocytes. Liebman further proposed a possible interconversion between eleocytes and colorless spherule amebocytes. Schinke (1950) reported that the colorless spherule amebocytes, after arising from connective tissue cells of the dermis, possibly differentiated into bladder and filiform amebocytes.

The diversity of opinions concerning the origin and interrelationships of echinoid coelomocyte types prompted the present autoradiographic investigation employing tritiated thymidine. Autoradiograms were prepared from tissues of sea urchins killed one hour after injection of tritiated thymidine, to determine the possible sites of coelomocyte (or coelomocyte precursor) proliferation. Autoradiograms were prepared from coelomocytes withdrawn from urchins at increasing time intervals after injection to demonstrate the possible transformation of labeled precursor cells into initially-unlabeled coelomocyte types. The circulating coelomocytes and the cells of the parietal peritoneum were studied quantitatively, since they could be prepared without histological sectioning, and yielded more reliable tritium indices than cells in sections. Autoradiograms of tissues that required sectioning were studied qualitatively. Coelomocyte production was investigated in starved as well as in freshly collected urchins.

MATERIALS AND METHODS

All specimens of *Strongylocentrotus purpuratus* investigated were collected from tide pools near Yankee Point, California, and were sexually mature. The weight and sex of each freshly collected urchin are given in Table I. On the day of collection, these urchins were injected intracoelomically *via* the peristomial membrane with 0.2 μc. of tritiated thymidine per gram of fresh weight. One part of the aqueous solution of methyl-tritiated thymidine (obtained from New England Nuclear Corp., Boston, and having a specific activity of 6700 mc. per mM) was diluted with four parts of sea water before injection. The injected urchins were returned to containers of sea water for 50 minutes. Then coverslip preparations of their coelomocytes were made by the following method:

A 10% (w/v) aqueous solution of disodium ethylene-diaminetetraacetic acid (EDTA), adjusted to pH 8.1 by slow addition of solid NaOH, was used as an anticoagulant. A 1.0-ml. syringe (fitted with a 0.5-inch, 27-gauge needle) was filled with 0.2 ml. of the EDTA anticoagulant. The needle was introduced into the perivisceral coelom of an urchin by puncturing the peristomial membrane in a radial position, and a 0.2 ml.-sample of coelomic fluid was withdrawn. The contents of the syringe were then quickly ejected into a flat-bottomed shell vial (15 ml. capacity, inside diameter 22 mm., and height 5 mm.) containing 5 ml. of the EDTA anticoagulant and a circular coverslip 18 mm. in diameter. The shell vial was placed in a regular centrifuge head, and the coelomocytes were spun down onto the coverslip at 500 rpm for ten minutes. The coverslip was then removed from the vial, and the adhering coelomocytes were fixed in formalin vapor under a bell jar. After overnight fixation, the still-moist coverslip preparation was placed in two successive 10-minute baths of distilled water. The preparation was subsequently placed in a bath of 5% acetic acid (aqueous) for 30 minutes to remove all traces of acid-soluble tritiated material adsorbed to the slide. The coverslip was rinsed in distilled water, dried, and finally glued, cell-side-up, to a microscope slide with Permount.

TABLE I

Tritium indices of coelomocytes and peritoneal cells from urchins killed one hour after injection

Urchin weight	Sex	Blad.-fil. amebocyte		Col. sph. amebocyte		Eleocyte		Vibratile cell		Peritoneal cell	
		Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell
17.5 g.	M	3.3%	32	0.2%	18	1.4%	27	0.0%	—	1.6%	22
15.1 g.	F	5.5%	40	1.1%	30	3.6%	37	0.0%			
16.5 g.	M	1.5%	40	0.7%	19	2.6%	34	0.0%	—	1.2%	26
16.0 g.	F	2.5%	18	1.1%	5	1.9%	13	0.0%	—	1.8%	17
17.0 g.	M	1.0%	24	1.0%	19	1.4%	21	0.0%	—	0.4%	15
16.8 g.	F	1.6%	27	0.5%	12	1.2%	15	0.0%	—	0.6%	27
16.0 g.	M	0.9%	36	0.3%	16	0.2%	32	0.0%		1.1%	17
18.1 g.	M	0.5%	31	0.6%	11	0.4%	30	0.0%	—	0.5%	33
18.1 g.	F	3.7%	37	2.8%	17	5.1%	38	0.0%		1.3%	27
27.2 g.	M	3.0%	38	0.3%	14	2.7%	26	0.0%	—		
35.7 g.	F	3.7%	31	0.0%	—	3.7%	34	0.0%	—		
29.4 g.	F	1.7%	24	0.0%	—	1.4%	27	0.0%			
Average tritium index + standard deviation		2.4 ± 1.5%		0.7 ± 0.8%		2.1 ± 1.6%		0.0 ± 0.0%		1.1 ± 0.5%	

After the coelomocyte sample had been taken, each urchin was replaced in its container for 10 more minutes and then fixed in sea water-Bouin's fluid. The Bouin's fluid had completely decalcified the urchins within one week. After decalcification, the urchins were washed in water and dehydrated as far as 70% ethanol. At this point, samples of the following organs were dissected out for histological sectioning at 20 μ, 7 μ and 2 μ: gonad, axial organ, pharynx, esophagus, stomach, intestine, Polian vesicle with tooth, interradial body wall and radial body wall with radial nerve and water vascular elements attached. At this time, the parietal peritoneum with its supporting basement membrane was stripped off one interradial sector of body wall simply by seizing it with forceps and pulling. After a rinse in distilled water, the stripped peritoneum was placed on a microscope slide and teased with two pins until it was spread out in a flat sheet with no wrinkles. The sheet was mounted with the basement membrane facing the surface of the slide and the coelom-bordering epithelial cells up. Mesenteric strands (anchoring the gut to the body wall in intact urchins) arose from the coelomic side of the peritoneum and aided in its orientation. Once the peritoneum had dried, it became firmly attached to the slide.

The stripped preparations of parietal peritoneum, the coverslip preparations of coelomocytes and the sectioned tissues were covered with Kodak AR-10 autoradiographic stripping film. The peritoneum and coelomocyte preparations were exposed for three weeks at 4° C. The sectioned tissues were exposed for 8 and 21 weeks at 4° C. After photographic processing (six minutes in Kodak D-19 developer at 18° C.), the coelomocytes and peritoneal cells were stained through the film with a 0.1% aqueous solution of toluidine blue for one minute. The emulsion was then destained for several minutes in 70% ethanol. After a rinse in tap water, the stained autoradiograms were dried, and immersion oil was placed directly on the film to observe them. For quantification, a labeled cell was defined as a cell having four or more silver grains above its nucleus. For each urchin, a minimum of 1000 cells was counted for each cell type quantified. For each cell type, the *tritium index* equalled $\frac{\text{number of labeled cells}}{\text{total number of cells counted}} \cdot 100$. The average number of grains per labeled cell was recorded with each tritium index. The autoradiograms of the sectioned

TABLE II

Tritium indices of coelomocytes and peritoneal cells from starved urchins killed one hour after injection

Aug. 27 Urchin weight	Sex	Blad. fil. amebocyte		Col. sph. amebocyte		Elecocyte		Vibratile cell		Peritoneal cell	
		Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell
14.7 g.	M									0.1%	50
33.9 g.	M									0.3%	33
24.1 g.	F	0.3%	50	0.0%	—	0.0%	—	0.0%	—	0.2%	50
28.7 g.	M	0.5%	50	0.0%	—	0.0%	—	0.0%	—	0.1%	33
33.9 g.	M	0.7%	50	0.0%	—	0.0%	—	0.0%	—	0.1%	20
19.2 g.	M	0.2%	40	0.0%	—	0.0%	—	0.0%	—	0.2%	31
21.4 g.	M	1.0%	50	0.7%	50	0.1%	50	0.0%	—	1.1%	36
34.3 g.	F	0.9%	34	0.5%	17	0.5%	34	0.0%	—	0.3%	30
22.8 g.	F	1.3%	50	0.0%	—	0.0%	—	0.0%	—		
22.3 g.	M	1.6%	50	0.0%	—	0.0%	—	0.0%	—		
Average tritium index ± standard deviation		0.8 ± 0.5%		0.2 ± 0.3%		0.1 ± 0.2%		0.0 ± 0.0%		0.3 ± 0.3%	

tissues were stained through the emulsion with toluidine blue or with nuclear fast red by the procedure of Montruil-Langlois (1962), omitting the counterstain.

Circulating coelomocytes from three uninjected donor urchins were exposed to tritiated thymidine *in vitro*. Each 30-g. donor was treated in the following way: By peristomial puncture, 0.2 ml. of perivisceral coelomic fluid was taken into a syringe containing 0.2 ml. of the EDTA anticoagulant at 14° C. The contents of the syringe was then ejected into a shell vial containing a circular coverslip and 5 ml. of the EDTA anticoagulant at 14° C. One μc. of tritiated thymidine in 0.01 ml. of distilled water was then added to the shell vial, and its contents were kept at 14° C. for 20 minutes. At least some of the cells collected in this way and subjected to washing with the EDTA anticoagulant are capable of proliferation in tissue culture (Phillips and Farmanfarmaian, unpublished data). Therefore, exposure to the EDTA anticoagulant does not appear to affect the viability of the cells. Autoradiograms of the *in vitro*-labeled coleomocytes were prepared by the methods already described, the only difference being that they were exposed for eleven days rather than three weeks.

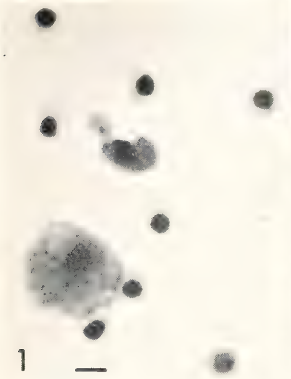
TABLE III

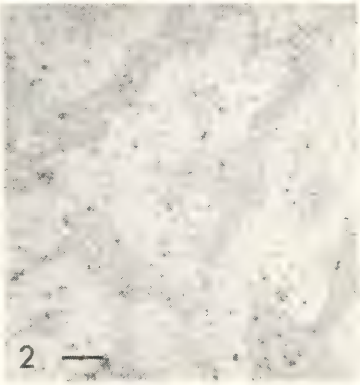
Tritium indices of coelomocytes from three urchins serially sampled following a single injection of tritiated thymidine: the average number of grains per labeled cell is given in parentheses

		Interval from injection to sampling			
		1 hour	24 hours	29 days	57 days
Sea urchin A	Blad.-fil. amebocyte	3.0% (38)		5.2% (21)	4.6% (17)
	Col. sph. amebocyte	0.3% (14)		4.9% (13)	7.9% (13)
	Eleocyte	2.7% (26)		4.8% (19)	5.9% (21)
	Vibratile cell	0.0% (—)		1.2% (17)	2.7% (16)
Sea urchin B	Blad.-fil. amebocyte	3.7% (31)	3.9% (31)		5.5% (19)
	Col. sph. amebocyte	0.0% (—)	0.1% (5)		5.0% (13)
	Eleocyte	3.7% (34)	1.9% (32)		6.9% (22)
	Vibratile cell	0.0% (—)	0.0% (—)		0.7% (15)
Sea urchin C	Blad.-fil. amebocyte	1.7% (24)	5.9% (31)	6.0% (26)	
	Col. sph. amebocyte	0.0% (—)	1.0% (23)	6.3% (11)	
	Eleocyte	1.4% (27)	5.7% (28)	8.8% (21)	
	Vibratile cell	0.0% (—)	0.0% (—)	0.3% (20)	

Each of ten urchins, which had been starved in the laboratory from April 19 to August 27, 1963, was injected with tritiated thymidine on August 27 and killed one hour later. The weight and sex of each starved urchin are given in Table II. The urchins were injected and sampled by the procedures already described for the freshly collected urchins. Coelomocytes, peritoneal cells, and other tissues were prepared for autoradiography by the procedures already given. The only difference was that the amount of perivisceral coelomic fluid withdrawn was increased from 0.2 ml. to 0.8 ml., since the concentration of coelomocytes in the coelomic fluid was lowered by the 130-day starvation.

The perivisceral coelomic fluid from three urchins (A, B and C of Table III) was serially sampled following a single injection of tritiated thymidine. The urchins were injected and sampled by the procedures already described for the freshly collected urchins. From each urchin, a 0.2-ml. sample of coelomic fluid was withdrawn one hour, 24 hours, 29 days and 57 days after the initial injection of tritiated thymidine. Autoradiograms were then made from the coverslip preparations of coelomocytes. Throughout the experiment, the three urchins were fed continuously on the alga, *Macrocystis*. Each of the 30-g. urchins gained about 5 g. during the 59 days of the experiment. After the final sampling of coelomocytes, the three urchins were killed, and their peritoneal cells and other tissues were prepared for autoradiography by the procedures already described.





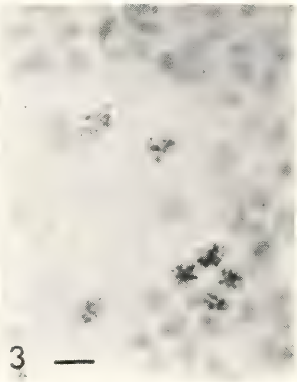


FIGURE 1. A toluidine blue-stained coverslip preparation of coelomocytes from the perivisceral coelom of *S. purpuratus*. In the field are seven despherulated eleocytes, one colorless spherule amebocyte (with a cytoplasmic process extending toward the upper left), one swollen vibratile cell and one bladder-filiform amebocyte (at lower right). The scale line is 5 μ long.

FIGURE 2. A stripped preparation of interradial parietal peritoneum showing the regions of sparsely distributed nuclei and the regions of closely packed cells in surface view. Toluidine blue. The scale line is 30 μ long.

FIGURE 3. A toluidine blue-stained autoradiogram of a stripped preparation of the parietal peritoneum from an urchin killed 57 days after injection of tritiated thymidine. The plane of the silver grains is in focus. The scale line is 5 μ long.

It was important to correlate the fixed and stained coelomocyte types, seen in coverslip preparations, with the living coelomocyte types described by Boolootian and Giese (1958). To accomplish this, a cross was scratched on one side of a circular coverslip, and coelomocytes were spun down onto the cross-bearing surface by the method already described. Prior to fixation, the living coelomocytes in the field with the cross were photographed. After fixation, the coelomocytes in the field with the cross were relocated and photographed a second time. The preparation was then stained in toluidine blue, and the same coelomocytes were photographed a third time.

RESULTS

The photographic correlation of the living coelomocyte types with the fixed and stained coelomocytes demonstrated that the vibratile cells, eleocytes, and colorless spherule amebocytes could be reliably identified in autoradiograms. The vibratile cells swell when fixed and flatten into discs 6 μ to 20 μ in diameter when dried onto the slide. Toluidine blue stains the vibratile cell nuclei light blue and the cytoplasm (which contains an acid mucopolysaccharide) violet to purple. The flagellum is rarely visible in stained vibratile cells (Fig. 1).

Colorless spherule amebocytes retain their spherules when fixed (Fig. 1). Toluidine blue stains their nuclei dark blue and their spherules light green. When fixed in formalin vapor, most but not all of the circulating eleocytes lose their spherules. Eleocytes without spherules typically have round nuclei 4 μ in diameter which stain dark blue with toluidine blue; their cytoplasm does not stain (Fig. 1). The eleocytes which retain their spherules are often swollen to the size of the vibratile cell in Figure 1, and toluidine blue staining reveals large blue spherules (up to 3 μ in diameter) in their cytoplasm. It is probable that the circulating eleocytes which retain their spherules when fixed are identical to the orthochromatic coelomocytes described wandering in the gut wall of *S. purpuratus* (Holland and Nimitz, 1964). The bladder amebocytes, filiform amebocytes, fusiform corpuscles and hyaline hemocytes of Boolootian and Giese (1958) could not be reliably distinguished from one another when fixed and stained. For the purpose of this investigation, these four cell types are considered to be a single type, the bladder-filiform amebocyte (Fig. 1). The fixed and stained bladder-filiform amebocytes are variable in shape and size (5 μ to 10 μ in diameter exclusive of cytoplasmic processes). They typically have a lightly staining nucleus and a basophilic cytoplasm which is often, but not always, extended into processes.

The data gathered from autoradiograms of circulating coelomocytes withdrawn from freshly collected urchins 50 minutes after injection of tritiated thymidine are presented in Table I. The circulating bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes synthesize DNA, while the circulating vibratile cells do not. The average tritium indices for the circulating bladder-filiform amebocytes, colorless spherule amebocytes, eleocytes and vibratile cells labeled *in vivo* are 2.4%, 0.7%, 2.1%, and 0.0%, respectively. Average tritium indices for the bladder-filiform amebocytes, colorless spherule amebocytes, eleocytes and vibratile cells removed from three urchins and labeled *in vitro* are 2.8%, 0.2%, 1.5% and 0.0%, respectively. Thus, the circulating coelomocytes labeled *in vitro* immediately after removal from the urchin have tritium indices similar to those of Table I. These results rule out the possibility that all the labeled coelomocytes of Table I incorporated tritiated thymidine outside the coelomic circulation as they were finishing DNA synthesis and then entered the coelomic fluid in the 50 minutes between injection and sampling.

Table I shows a striking variability in coelomocyte tritium indices between individual urchins. Since all urchins listed were treated at approximately the same time of day, the variability is not due to a diurnal cycle of fluctuating tritium indices. Such fluctuations in tritium indices do occur in some cell types in mice (Pilgrim *et al.*, 1963). It also appears from Table I that, within the coelomic fluid of each individual, the tritium indices for the bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes are correlated—when one is high, the other two tend to be high also. To test the validity of this impression, a correlation coefficient analysis was performed according to instructions in Moroney (1958, pp. 336–340). The analysis, the details of which are given in Holland (1964), demonstrates that the correlation is significant at the 5% level. This significant correlation suggests that DNA synthesis in the circulating bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes is under the control of one or more common factors present in the plasma of the coelomic fluid. Possible controlling factors might include either hormonal substances or nutrients necessary for DNA synthesis.

Presumably, DNA synthesis is eventually followed by mitosis of the circulating coelomocytes. In autoradiograms of coelomocytes withdrawn from urchins 24 hours after injection of tritiated thymidine, a number of labeled coelomocytes have two groups of silver grains above them, indicating that anaphase or telophase is occurring in the cell beneath. However, no chromosomes can be seen beneath the grains. Unequivocal mitotic figures also can not be detected in coverslip preparations of coelomocytes fixed in sea water-Bouin's fluid or Carnoy's acetic alcohol and stained with hematoxylin. It is apparent that some, and perhaps all, of the circulating coelomocytes which synthesize DNA subsequently divide in the coelomic fluid, but the mitotic figures are very difficult (if not impossible) to detect by ordinary histological methods. This undoubtedly explains the failure of earlier workers to detect mitosis in sea urchin coelomocytes (Kollmann, 1908; Yeager and Tauber, 1935; Schinke, 1950). It is interesting to note that Phillips and Farmanfarmaian (unpublished data) found that coelomocytes taken from the perivisceral coelom of *S. purpuratus* proliferated when cultured for several weeks *in vitro*. When coverslip cultures were fixed in Kahle's alcoholic formolacetic acid and stained with hematoxylin, some of the cultured coelomocytes showed all stages of mitosis with distinct chromosomes.

In addition to coelomocyte tritium indices, Table I presents tritium indices for the cells of the parietal peritoneum labeled for one hour *in vivo*. The interradial parietal peritoneum (Fig. 2) consists of regions of closely packed cells with elongate, often reniform, nuclei and regions of sparsely distributed, oval nuclei; cells of both regions are flagellated. The regions of closely packed cells run in interconnecting tracts, which may be seen in living, as well as fixed, stripped preparations (living peritoneum may be stripped from the dermis in small pieces). A one hour's exposure to tritiated thymidine labels some cells in both regions. For calculating the tritium index, cells of both regions were counted together. Some, and presumably all, of the peritoneal cells which synthesize DNA subsequently divide, since autoradiograms of peritoneum from urchins killed 57 days after injection of tritiated thymidine show that labeled cells (in both regions) frequently occur in groups of two to four (Fig. 3). Groups of more than four labeled cells also occur, but are less easily identified as progeny of a single initially-labeled cell.

In addition to cells of the parietal peritoneum, cells of all other tissues named by earlier workers as sites of coelomocyte production synthesize DNA. Autoradiograms of sectioned tissues from freshly collected urchins killed one hour after injection show some labeled cells in the radial parietal peritoneum, in the visceral peritoneum, in the epithelium lining all parts of the water vascular system, in the axial organ, in the Polian vesicles, in the hemal strands and in the dermal connective tissues of the body wall. Furthermore, there is DNA synthesis by some of the eleocytes and colorless spherule amebocytes wandering in the body wall and gut wall. Cells of other tissues also synthesize DNA, but are not listed here since they have not been implicated in coelomocyte production. All tissues named as sites of coelomocyte production still contain labeled cells in urchins killed 57 days after injection.

The tritium indices of coelomocytes and peritoneal cells from the starved urchins killed one hour after injection are presented in Table II. The average tritium indices of bladder-filiform amebocytes, eleocytes and peritoneal cells are significantly lower in starved than in freshly collected urchins. Due to large standard deviations, the average tritium index of colorless spherule amebocytes is not significantly lower (at the 5% level) in starved than in freshly collected urchins. The circulating vibratile cells, in the starved as in the freshly collected urchins, never synthesize DNA. Autoradiograms of sectioned tissues from the starved urchins showed that a few cells of all regions implicated in coelomocyte production synthesize DNA. The average number of grains per labeled cell is higher in starved than in freshly collected urchins, perhaps because a smaller number of cells synthesizing DNA results in less competition for the injected tritiated thymidine, perhaps because of a lesser dilution of the tritiated thymidine by unlabeled endogenous thymidine.

After four months' starvation, protein in the perivisceral fluid of *S. purpuratus* declined from .37 mg. per 100 ml. to .14 mg. (L. Holland, unpublished). After 41 days' starvation, lipid in the perivisceral fluid declined from 25 mg. per 100 ml. to 15 mg. and carbohydrate from 2 mg. per 100 ml. to 1 mg. (A. Lawrence and J. Lawrence, unpublished).

The tritium indices for the coelomocytes of the serially sampled urchins are presented in Table III. In donors A and C, labeled vibratile cells appear in the circulation sometime between 24 hours and 29 days after injection of tritiated thymidine. The 29-day sample for donor B was not countable, but labeled vibratile cells had entered its circulation by 57 days after injection. Therefore, some proliferating cell type must be differentiating into vibratile cells. The time course for this differentiation is on the order of several days at the least and several weeks at the most. The serial results for the bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes are not very consistent. In most cases, the tritium index rises with time, but sometimes it drops. The striking rise in the tritium index of all three coelomocyte types of urchin C between one hour and 24 hours is unexpected and will be discussed below. The average number of grains per labeled cell usually falls slowly with time but in several instances remains constant or rises.

DISCUSSION

The circulating vibratile cells of *S. purpuratus* are mature, non-proliferating cells, which must necessarily differentiate from one or more proliferating cell populations somewhere in the sea urchin. Of the many possible sources of vibratile cells, the cells of the parietal peritoneum are the most likely. The cytoplasm of some cells of the parietal peritoneum is filled with granules which stain violet with toluidine blue (*i.e.*, show B-metachromasia) and which may be a mucopolysaccharide similar to the one found in mature vibratile cells. Such a mucopolysaccharide does not occur in the other cell populations which have been implicated in coelomocyte production. Furthermore, as Liebman (1950) pointed out, each cell of the peritoneum bears a single flagellum, which could be a precursor of the longer flagellum of a vibratile cell. Since the vibratile cell is a non-dividing, differentiated cell type, it is most unlikely that it transforms into other coelomocyte types as Liebman claimed in 1950.

The lack of DNA synthesis in circulating vibratile cells, as well as the appearance of labeled vibratile cells in the circulation in the weeks following injection, does not support the suggestion of Cuénot (1948, p. 149) that vibratile cells are not urchin cells at all, but are parasitic flagellate protozoans of the genus *Oikomonas*. The conclusion that the vibratile cells are sea urchin cells and not parasites is further supported by Boolootian's 1962 report that the perivisceral fluid in all individuals of twelve different species of regular echinoids contained vibratile cells. Such universal distribution and lack of specificity is not characteristic of a parasite.

Some of the bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes of *S. purpuratus* synthesize DNA and apparently divide. All new coelomocytes could arise by division of pre-existing coelomocytes, which would thus constitute self-maintaining cell populations. Cuénot (1897, p. 185) proposed just such a system for replacement of circulating cells of some arthropods, some annelids and some molluscs. However, all circulating and tissue coelomocytes synthesizing DNA could go through only one or a few growth-duplication cycles and thus constitute an amplification compartment of a cell system which must necessarily have a self-maintaining compartment somewhere in the urchin. This self-maintaining, ultimate source of coelomocytes could be one or more of the tissues which have been named as sites of coelomocyte production, since cell proliferation occurs in all of them. There are many possible schemes between these extremes. For example, only one of the three coelomocyte types could be a self-maintaining cell population. Furthermore, tissue coelomocytes might have a different renewal scheme from circulating coelomocytes of the same type. There is also the possibility (which was neither proved nor disproved in the present investigation) that one type of coelomocyte may differentiate into other types. The present lack of information on these points makes an unambiguous interpretation of Table III impossible.

Interpretation of the data in Table III is further hampered by the lack of information on the possible inter-changes of circulating and tissue coelomocytes and on the related question of whether or not the circulating coelomocytes are in a steady-state. It is known that large numbers of coelomocytes enter and leave the coelomic circulation in stressed urchins which have had all or part of their coelomic fluid removed (Schinke, 1950; Boolootian and Lasker, 1964). In the present investigation, urchins A, B, and C of Table III were probably stressed to some extent by withdrawal of 0.2 ml. (5% to 10%) of their coelomic fluid at each sampling. The sampling of urchin C one hour after injection could well have induced an immigration of tissue coelomocytes (having high tritium indices) into the coelom between one and 24 hours after injection.

In adult mammals, a renewing cell population has an initially-high tritium index; with time, the tritium index and the average number of grains per labeled cell fall rapidly (Messier and Leblond, 1960). An expanding cell population in mammals has an initially-low tritium index; with time, the tritium index and the average number of grains per labeled cell tend to persist. The data in Table III demonstrate that sea urchin coelomocytes resemble expanding, rather than renewing cell populations, of mammals. Indeed, most of the cell populations of adult sea urchins have the characteristics of expanding cell populations. In the adult urchin, only the gametogenic cells of the male during the ripe season and the tooth cells have the characteristics of renewing cell populations (Holland, 1964).

The starved urchins of Table II showed no weight increase during starvation, although they appeared healthy in every other way. Presumably, the number of cells in their constituent cell populations was not increasing at the time of labeling on the last day of starvation. In most of the cell populations of the starved urchins, the few cells synthesizing DNA (33% of normal in the bladder-filiform amebocytes and 27% of normal in the peritoneal cells) are probably preparing to divide for replacement of cells lost from the populations and not for growth. Probably, even under normal conditions of growth, a minor component of proliferation in each expanding cell population is for replacement and not for growth. The reduction of tritium indices by starvation might be due to a reduction in the nutrients necessary for cell proliferation or to starvation-induced hormonal changes. Since it is unlikely that starvation shortens the DNA synthesis period, the lowering of the tritium indices is probably due to an increase in the duration of the growth-duplication cycle by a lengthening of the period of the interphase preceding the DNA synthesis period.

SUMMARY

1. Brief exposure to tritiated thymidine *in vivo* or *in vitro* labels some of the bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes circulating in the perivisceral coelomic fluid of the purple sea urchin; circulating vibratile cells are not labeled. Some colorless spherule amebocytes and eleocytes are also labeled while wandering in the body wall and gut wall.

2. Some, and presumably all, of the circulating coelomocytes which synthesize DNA subsequently divide in the circulation. It is not known if this proliferation constitutes all or only a part of production of new bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes in the sea urchin.

3. The tritium indices of the circulating bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes within each individual urchin are significantly correlated. This suggests that DNA synthesis in these circulating cells is under the control of one or more common factors.

4. Brief exposure to tritiated thymidine labels some cells in the visceral peritoneum, the parietal peritoneum, the epithelium lining all parts of the water vascular system, the axial organ, the Polian vesicles, the hemal strands and the dermal connective tissue of the body wall. These tissues have been named by previous authors as sites of coelomocyte production. The present investigation found no evidence for or against their participation in the production of bladder-filiform amebocytes, colorless spherule amebocytes or eleocytes.

5. In the weeks following injection of tritiated thymidine, labeled vibratile cells begin to appear in the coelomic fluid. The source of the vibratile cells is probably the parietal peritoneum. The vibratile cells thus constitute a sea urchin cell type and are not, as has been claimed, parasitic flagellates.

6. Serial autoradiographic data demonstrate that the coelomocytes (and most of the other tissues) of the young adult sea urchin have the characteristics of expanding, and not renewing, cell populations.

7. Starvation for several months causes a significant reduction in the tritium indices of the bladder-filiform amebocytes, eleocytes and cells of the parietal peritoneum.

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PHOSPHATASES OF MARINE ALGAE

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The phosphatases are enzymes important to life because of their ability to hydrolyze phosphate esters or to transfer phosphate from one organic group to another. Phosphomonoesterases hydrolyze monoesters of phosphoric acid,

$$RO - PO_3H_2 + H_2O \rightarrow ROH + H_3PO_4,$$

and are classified as acid- or alkaline-phosphatases depending on the pH at which maximum activity occurs. Other phosphatases hydrolyze diesters of phosphoric acid, pyrophosphates, or metaphosphates; unless otherwise stated, however, the term "phosphatase" will hereafter be used to designate only phosphomonoesterases. Constitutive enzymes are those always formed by cells, regardless of the medium in which they are grown. Induced enzymes are those produced by an organism only when it is exposed to a specific substrate; it then permits the organism to utilize this substrate. Repressible enzymes are those synthesized only when the concentration of a particular substance, a represser, becomes very low.

Phosphatases in algae have been reported by several workers recently. Brandes and Elston (1956), using histochemistry and electron micrography, reported the alkaline phosphatase of *Chlorella vulgaris* to be localized at the cell surface. Talpasayi (1962), studying the inhibition of acid phosphatases of algae by molybdenum, found that living cell suspensions showed more enzyme activity than broken cells. Eppley (1962) found that several species of intertidal algae hydrolyze pyrophosphate and assimilate a portion of it; most of the resulting orthophosphate remained in the medium, however. Galloway and Krauss (1963) found that pyrophosphate induced the production of pyrophosphatase in *Chlorella pyrenoidosa* cultures; cell walls from disrupted *Chlorella* contained most of this enzyme. Overbeck (1962) found that *Chlorella pyrenoidosa* cultures split pyrophosphate and glycerophosphate. He also reported that *Scenedesmus quadricauda* contained three phosphomonoesterases and three pyrophosphatases but they seemed to be inside the cell rather than at the surface and the substrates did not penetrate the cell wall. Living *Scenedesmus* did not assimilate pyrophosphate or phosphomonoesters rapidly even after seven weeks in the presence of these substrates. Price (1962) showed that 2 m*M* phosphate was sufficient to repress synthesis of the acid phosphatase of *Euglena gracilis*.

Many species of algae are capable of obtaining phosphorus from esters in order to sustain growth in the absence of orthophosphate (see review by Provasoli, 1958). We found that many species of marine algae produce alkaline phosphatase when they

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[2] Supported by U. S. Atomic Energy Commission Contracts AT(30-1)-1918 and AT(30-1)-3145.

become phosphorus-deficient. This enzyme hydrolyzed dissolved phosphate esters on or outside the cell; the cell then absorbed only the phosphate ion, leaving the organic moiety in the medium.

We are very grateful to Drs. R. R. L. Guillard, J. C. Hellebust, and C. A. Price for many stimulating discussions and suggestions. Dr. Guillard also generously furnished the pure cultures of marine algae; the *Chlamydomonas reinhardi* culture was obtained from the Harvard University laboratory of Dr. R. P. Levine through the courtesy of Miss J. C. Brungard. We are also grateful to Drs. J. H. Ryther, R. R. L. Guillard, and A. Torriani for reading and criticizing the manuscript.

METHODS

Stock axenic algal cultures were maintained in half-strength Medium *f* (Guillard and Ryther, 1962): 75 mg. $NaNO_3$, 5 mg. $NaH_2PO_4 \cdot H_2O$, 5 mg. FeEDTA, 15–30 mg. $Na_2SiO_3 \cdot 9H_2O$, 100 μg. thiamine·HCl, 0.5 μg. Biotin, 0.5 μg. vitamin B_{12}, 10 μg. $CuSO_4 \cdot 5H_2O$, 22 μg. $ZnSO_4 \cdot 7H_2O$, 10 μg. $CoCl_2 \cdot 6H_20$, 180 μg. $MnCl_2 \cdot 4H_20$, 6.3 μg. $Na_2MoO_4 \cdot 2H_2O$, 1 liter filtered sea water. *Chlamydomonas* spp. were furnished with NH_4Cl instead of $NaNO_3$ as the nitrogen source. Stock cultures were tested periodically for bacterial contamination by inoculating into sea water medium containing 0.1% tryptone. Phosphorus-deficient algae were produced by growing them in sea water medium containing double the concentration of all the above nutrients except phosphorus; it was reduced to 7 μ*M* or less. All cultures were grown at 20° C. except *Detonula confervacea* and *Thalassiosira nordenskioldii* which were grown at 5° C. Cells were counted using a Spencer "Bright Line" or a Palmer counting chamber. Cell dimensions were obtained with a calibrated ocular micrometer and cell volumes were calculated by formulae for similar geometric solids (sphere, cylinder, etc.).

Cellular protein was measured by the Folin-Ciocalteu phenol reagent by a method that we modified from Lowry *et al.* (1951). Algal suspensions were centrifuged for 5 minutes and the supernatant was discarded. The cells were resuspended in 5 ml. of 10% trichloroacetic acid (TCA) and held 10 minutes at 100° C. to precipitate protein and dissolve nucleic acids and other acid-soluble material. The precipitate was centrifuged and washed twice with 5 ml. of 10% TCA. The protein was redissolved in 0.5 ml. 1 N NaOH overnight, then 5 ml. of Lowry's Reagent D (50 parts 2% Na_2CO_3 plus 1 part 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium-potassium tartrate; mixed fresh daily) was added and the volume was made up to 6 ml. with distilled water. After 10 minutes 0.5 ml. 1 N Folin-Ciocalteu phenol reagent was added and the absorbance at 750 mμ was read 30 minutes later on a Beckman DU spectrophotometer. Protein standardization was performed on bovine serum albumin by the Lowry *et al.* (1951) procedure.

Enzyme activity was assayed by the rate at which nitrophenol accumulated during hydrolysis of *p*-nitrophenyl phosphate (NPP) at known hydrogen ion concentrations. The NPP stock consisted of 0.1 g. of NPP plus 2.5 g. $MgSO_4$ in 50 ml. distilled water. The pH range 3.0 to 6.8 was covered by 1 *M* citric acid-potassium citrate solutions and the range 7.2 to 9.8 by 1 *M* Tris (hydroxymethyl)amino methane·HCl solutions. The actual pH value of filtered sea water containing these buffer solutions was measured with a Beckman Model 76 hydrogen ion meter. For each analysis, the algal culture: NPP stock: citrate buffer stock: autoclaved-sea water diluent ratios were usually about 1:1:1:30. In order to obtain adequate alkaline buffering however, it was necessary to use twice as much Tris, giving 1:1:2:30 ratios. Unless otherwise noted, all measurements of enzyme activity were performed on whole, living cells in darkness or dim light at 19°–21° C. After incubation, sufficient pH 10 Tris buffer was added to the low pH samples to bring out nitrophenol color (pH 9) and the absorbance of nitrophenol was measured in a Beckman DU spectrophotometer at 410 mμ in 1-cm. or 10-cm. cells. Enzyme activity was calculated from the nitrophenol-time regression; one "Unit" represents the amount of enzyme that causes a change in optical density of 0.001 per minute in a 1-cm. cell.

The rate of assimilation of phosphorus by algae from glucose-6-phosphate (G-6-P), adenosine monophosphate (AMP), and α-glycerophosphate (α-GP) was measured by periodically determining the phosphorus content of the algal cells. Two ml. of cultures of phosphorus-deficient cells were added to 8 ml. of filtered, autoclaved sea water (pH ~ 8) containing 0.1–0.2 μmole of one of these three esters in centrifuge tubes. Periodically, duplicate tubes were centrifuged for 5 minutes, the supernatant was discarded, and the cellular phosphorus was determined by measuring the orthophosphate after hydrolysis in 0.55 N H_2SO_4 at 140° C. for 5 hours (Ketchum *et al.,* 1955). Total phosphorus initially present in the sea water, the algal cultures, and the three substrates was measured the same way. Inorganic phosphate of sea water was measured by the method of Wooster and Rakestraw (1951).

Rate of uptake of the glucose from G-6-P was determined from the decrease in radioactivity of a C^{14}-labeled solution. Ten μc. of the Ba salt of D-glucose-1-C^{14}-6-phosphate were dissolved in 3 ml. distilled water and converted to the Na salt by passage through a 7 × 30-mm. strong cation exchange column (Dowex 50) in the Na^+ form; the column was rinsed with 2 ml. of distilled water. This was diluted 1:100 to obtain a suitable count-rate and 0.25 ml. was added to centrifuge tubes containing autoclaved sea water (pH ~ 8), G-6-P carrier, and phosphorus-deficient algae as described above. Subsamples of this suspension were periodically centrifuged and samples of the supernatant were dispensed (0.2–0.5 ml.) onto planchets, dried, and the C^{14} counted with a windowless gas flow detector. The same volumes of the original whole suspension were also dried, and the initial count rate thus obtained had geometry and self-absorption factors similar to the supernatant samples.

RESULTS

Phosphatase synthesis by deficient algae

Alkaline phosphatase is produced abundantly when algae are grown in a phosphate-limited medium, or stated conversely, its production is repressed in phosphorus-sufficient algae (Table I). In each species the enzyme activity of P-deficient cells was at least 30 times greater than in cells limited by vitamins, iron and trace metals, nitrogen, or silicon. (*Phaeodactylum* has no requirement for vitamins or silicon, nor *Coccolithus* for silicon; hence these experiments were not performed.)

Table I

Effect of nutrient limitation on production of alkaline phosphatase. Cultures were grown in Medium f deficient in one of the 5 types of nutrients until cell numbers increased less than 10% per day. Enzyme activity (Units/10^7 cells) was measured at 20°–25° C. All measurements were made at pH 8.0, although later it was found that this was not at the pH optimum for the phosphatase of these species.

Nutrients Omitted:	Thiamin Biotin, B_{12}	Fe, Cu, Zn, Co, Mn, Mo	N	Si	P
Phaeodactylum tricornutum	—	1.1	2.1		62
Cyclotella nana (clone 13-1)	0	0.03	0.10	0.03	6.3
C. nana (clone 3H)	0	0.17	0.02	0.21	12
Skeletonema costatum	0.04	0.27	0.27	0.13	12
Coccolithus huxleyi	0.05	0.04	0	—	59

The low enzyme activities in P-sufficient cultures are not the result of inhibition of enzyme activity by phosphate in the medium. In one experiment we measured the phosphatase activity of *Coccolithus huxleyi* in the presence of various orthophosphate concentrations and found a nearly linear inhibition of activity amounting to 80% at 1.0 mM. However, the initial phosphate concentration of Medium *f* is

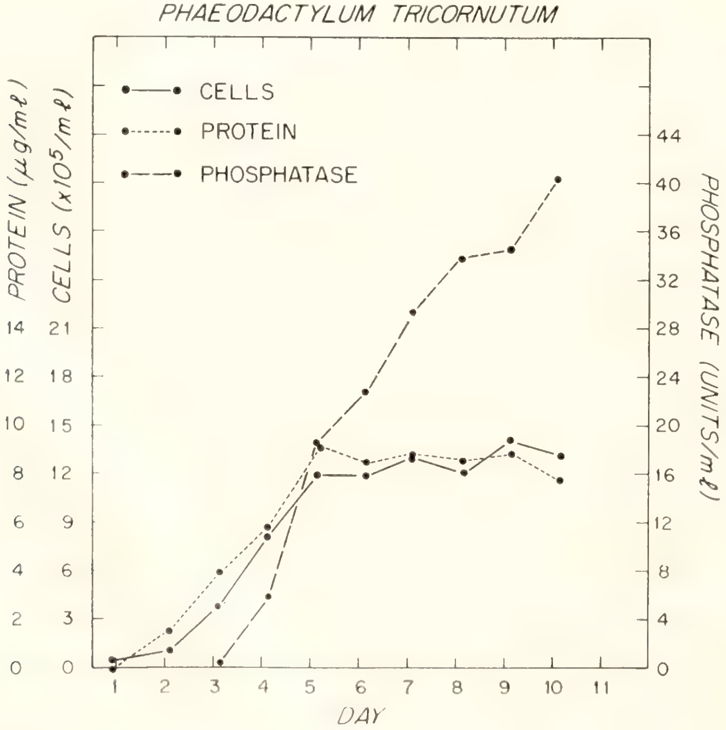

Figure 1. Changes in cell numbers, protein content, and enzyme activity during growth of a *Phaeodactylum tricornutum* culture: 15 liters stirred by constant aeration; 2.5 μM P; 25° C.; 300 f.c. illumination; enzyme measured at pH 9.7.

only 72 μM and the inhibition of enzyme activity should have only been about 6% even if no phosphate had been assimilated by the cells. As discussed below, the amount of phosphatase in a culture increases with time; thus, comparisons of the values for the different species in Table I are unwarrantable.

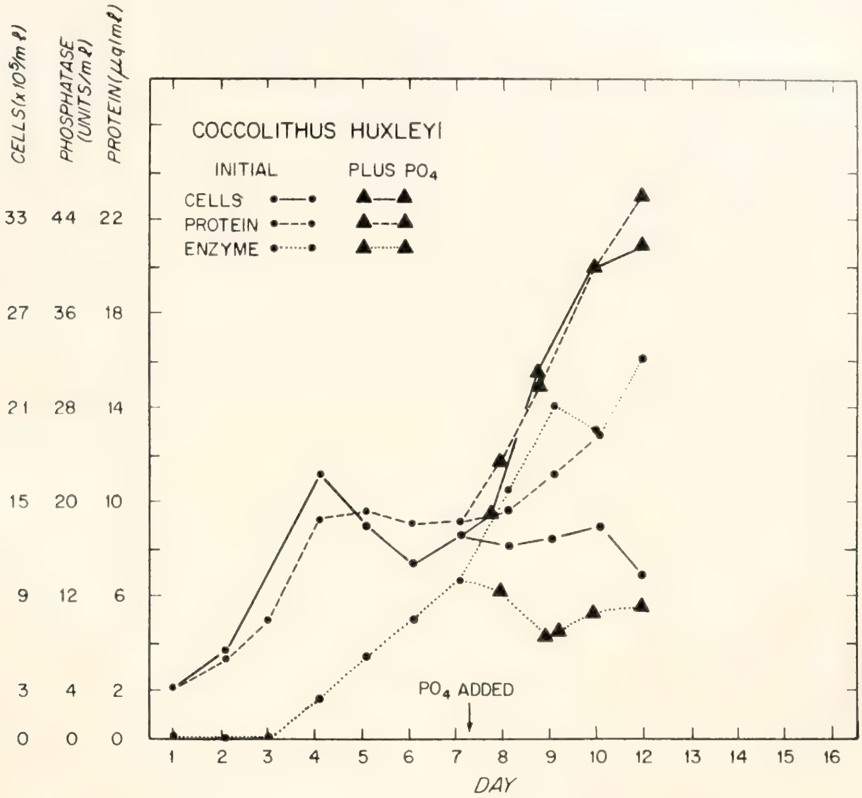

FIGURE 2. Changes in cell numbers, protein content, and enzyme activity during growth of a *Coccolithus huxleyi* culture: 1 liter shaken several times daily; 3.7 μM P; 20° C.; 300 f.c. illumination; enzyme measured at pH 9.7; phosphate added on day 7 increased the content of the medium by 25 μM P.

Time course of phosphatase production

The production of alkaline phosphatase in *Phaeodactylum* began when the average cell still had enough phosphorus to divide at least once more, *i.e.*, about two days before cell division and protein synthesis stopped (Fig. 1). Enzyme production continued at a nearly linear rate at least 5 days after the apparent halting of net protein production.

The same pattern of initiation of enzyme production just before growth stopped was repeated in two experiments with *Coccolithus*, the results of one being shown in Figure 2. Repression of enzyme synthesis by phosphate was confirmed when this nutrient was added to half of the culture on day 7, the remaining half representing a control. Phosphate stopped enzyme production whereas cell numbers and cellular protein increased again at rates comparable to those on days 1 to 3.

The cellular phosphorus per unit volume of P-deficient marine algae ranged from 1 to 15 × 10^{-17} mole P/μ^3 in species of different sizes (Table II). Cellular protein per unit volume, however, differed by less than a factor of 5 among these same species. Some variability in both P- and protein-content might arise from calculation of cell volume, due to irregularity of cell shape and uncertainty as to the thickness of the enveloping frustules, coccoliths, or cell walls. A large vacuole would also tend to lower the amount of P or protein per unit whole cell volume. The P:Protein ratios calculated from Table II were more nearly constant, from 230 to 790 μmole P/g. protein. Some algae synthesized alkaline phosphatase much faster than others when compared on the basis of cellular volume (Table II). Further work will undoubtedly show conditions under which higher rates occur in some of these algae, but repeated experiments indicate that, under our conditions, there are obvious differences. *Coccolithus, Cricosphaera, Phaeodactylum,* and *T. fluviatilis* repeatedly showed rapid enzyme production when phosphorus-deficient.

TABLE II

Cell volume, phosphorus, protein, and maximum observed rate of production of phosphatase in phosphorus-deficient marine algae

	Cell volume (μ^3/cell)	Cell P (10^{-17} mole/μ^3)	Cell protein (10^{-14} g/μ^3)	Enzyme production rate (10^{-8} units/μ^3·day)
Chrysophyceae				
Isochrysis galbana	115	1.0	4.3	6
Cricosphaera carterae	1240	1.7	5.8	16
Coccolithus huxleyi	73	2.2	6.2	26
Bacillariophyceae				
Phaeodactylum tricornutum	53	3.2	5.7	10
Chaetoceros simplex	120	1.6	5.9	6
Thalassiosira fluviatilis	1300	2.9	7.4	10
Cyclotella caspia	210	3.5	7.6	6
C. nana (clone 13-1)	140	3.4	10	0.2
C. nana (clone 3-H)	85	2.7	7.8	2
C. cryptica	640	4.3	15	11
Nitzschia closterium	84	15	19	3
Skeletonema costatum	160	8.0	12	0.5
Dinophyceae				
Amphidinium carteri	660	2.9	10	0.4

Optimum pH

The rate at which phosphorus-deficient algae hydrolyzed NPP was markedly pH-dependent. The graphs of five representative species (Fig. 3) show that significant enzyme activity generally extends one or more pH units on either side of the peak. Some species have more than one peak. It should be remembered that the absolute height of the alkaline-phosphatase peak is a function of both the length of time since the cells became P-deficient and specific physiological factors. For this reason emphasis is not placed on the amount of enzyme of one species compared to that of others.

Twenty-nine clones (at least 25 species) of marine algae were tested to determine the pH at which their phosphatases were most active (Table III). Two species had maximum rates less than 0.6 Unit/ml. of culture and are not included in the table. All chrysophytes and diatoms (Bacillariophyceae) had an abundant enzyme with activity at pH 8.6 or higher. *Detonula* had a slightly higher peak at pH 6 than at pH 9, but this might have only been the result of its lower metabolic rate at 5° C.; the alkaline phosphatase activity presumably would overtake that of the acid in another few days. *Thalassiosira nordenskioldii,* the only other species grown at 5° C., was also relatively low in alkaline phosphatase, whereas *T. fluviatilis* was similar to other diatoms. The three clones of *Rhodomonas,* although originally isolated from different places, had almost identical pH graphs (see example, Fig. 3) and showed clearly that large amounts of alkaline phosphatase are not produced by *Rhodomonas* under these conditions. Their enzymes are probably constitutive; a repeat experiment with clone F-5A showed essentially the same amount of alkaline phosphatase as the first. The blue-green alga, *Oscillatoria,* produced alkaline phosphatase, whereas *Coccochloris* sp. (clone Syn)

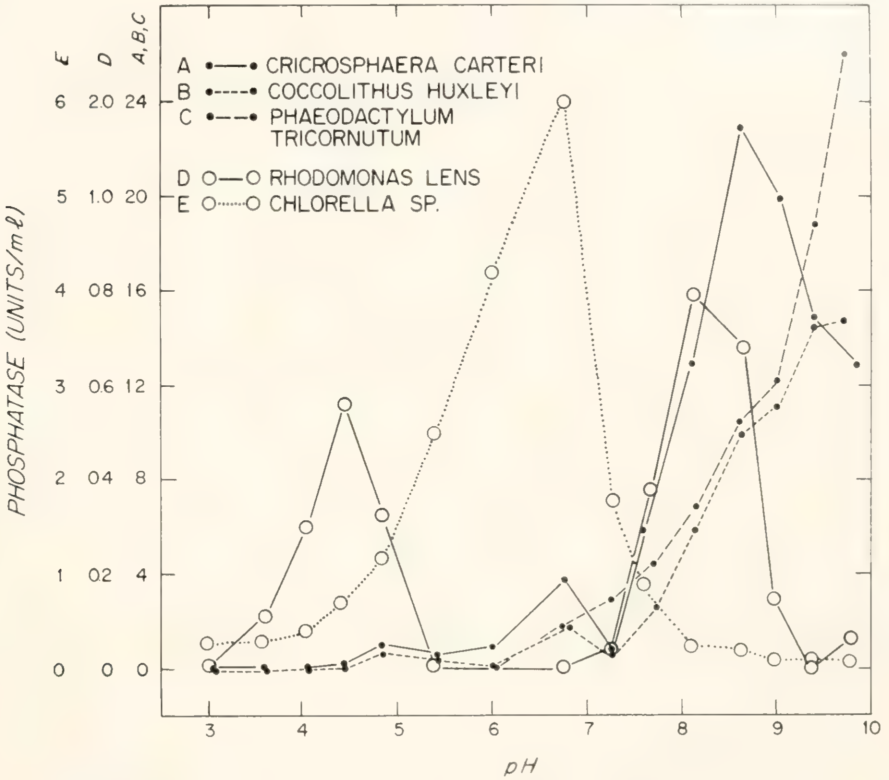

FIGURE 3. Five examples of phosphomonoesterase activity from pH 3 to 10.

(not shown) did not. *Gymnodinium nelsoni* is interesting because both the acid and the alkaline phosphatases more than doubled during four days of P-deficiency. This is our only example of a phosphorus-repressible acid phosphatase. The two species of *Chlamydomonas* were the only green algae with significant amounts of alkaline phosphatase. Clone 0–5 was extremely rich; furthermore it had two alkaline peaks, the second being 475 Units/ml. at pH 8.6. The chlorophyte, *Dunaliella tertiolecta* (not shown), was tested twice without acid or alkaline phosphatase being detected. *Pyramimonas* was also tested twice. *Chlorella* sp.

TABLE III

The pH optima of algal phosphatases. The clone designation of each of the algae is in parentheses after the name. The habitat type from which clones were obtained and isolated are: E, estuarine or rock pool; N, neritic; and O, oceanic. One Unit/ml. is the enzyme activity per ml. of culture that causes a change in O.D. of 0.001 per minute in a 1-cm. cell.

	pH Optima				
	Acid		Alkaline		Habitat
	Units/ml.	pH	Units/ml.	pH	
Chrysophyceae					
Isochrysis galbana (Iso)	1.9	6.8	28	9.8	E
Monochrysis lutheri (Mono)	--	- -	8	9.7	E
Cricosphaera carterae (Cocco II)	3.9	6.8	23	8.6	N
Coccolithus huxleyi (BT-6)	1.8	6.8	15	9.7	O
Bacillariophyceae					
Phaeodactylum tricornutum (Phaeo)	–		26	9.7	E
Melosira sp. (T-5)	0.6	6.8	6	9.7	E
Cyclotella nana (3H)			2.6	9.8	E
C. cryptica (O-3A)		-	37	9.7	E
Detonula confervacea (D. con.)	1.1	6.0	0.7	9.0	E
Thalassiosira fluviatilis (Actin)	0.6	6.8	18	9.8	N
T. nordenskioldii (T. nord.)	—	—	1.3	9.0	N
Nitzschia closterium (N. clost.)	–	- -	2.7	9.8	N
Skeletonema costatum (Skel)			0.7	8.6	N
Chaetoceros sp. (*simplex?*) (BBsm)	2.3	6.8	18	9.7	O
Cyclotella caspia (10-5)	1.5	6.8	12	9.8	O
C. nana (13-1)			1.5	9.8	O
Cryptophyceae					
Rhodomonas (?) (3C)	0.5	4.4	0.8	8.6	E
Rhodomonas (?) (F-5A)	0.5	4.4	0.6	8.6	E
Rhodomonas lens (Rhodo)	0.6	4.4	0.8	8.1	O
Cyanophyceae					
Oscillatoria (?) *woronichinii* (Sm 24)	0.6	6.8	3.7	9.0	E
Dinophyceae					
Amphidinium carteri (Amphi 1)			9	9.0	E
Gymnodinium nelsoni (GSBL)	0.8	5.8	0.3	9.8	E
Chlorophyceae					
Chlorella sp. (580)	6	6.8	- -	—	E
Stichococcus sp. (GSB Sticho)	2.1	4.8		—	E
Pyramimonas sp. (Pyr 2)	0.9	4.8		--	E
Chlamydomonas sp. (0-5)			650	7.6	E
Chlamydomonas sp. (1-17)			7.5	8.6	E

had more acid phosphatase than any other alga tested, but even this was low relative to the alkaline phosphatases of many other species. Except for *Gymnodinium,* acid phosphatase production did not seem to begin when these algae became P-deficient as was reported in *Euglena gracilis* by Price (1962). The relative constancy in the amount of acid phosphatase within the species tested (Table III) argues that these enzymes are constitutive. It is possible that the alkaline phosphatases found in only small amounts in certain algae are also constitutive; further study of these species is necessary.

The phenomenon of alkaline phosphatase production when phosphate-deficient was found in algae from estuarine, neritic, and oceanic habitats (Table III).

Uptake of phosphate esters

When P-deficient cultures were exposed to three different phosphate esters, all esters were assimilated at the same rate by each algal species (Fig. 4). The intercept at time-zero is the amount of phosphorus in the algae at the beginning of each experiment; the somewhat accentuated uptake during the first 5–8 minutes simply reflects the amount of inorganic phosphate immediately available in the diluting sea water. The curves from 5 to 125 minutes represent uptake of phosphorus from the esters. In *Phaeodactylum* the uptake rate remained almost constant during this time, even though at 124 minutes about 80% of the G-6-P had been assimilated. The initial levels of the other substrates were higher, and such a high proportion was not assimilated during this period. The amount of phosphorus per *Phaeodactylum* cell may be calculated from Figure 4. The final value,

$$\frac{11 \times 10^{-6}\ \text{mole P/l.}}{5.7 \times 10^{8}\ \text{cells/l.}} = 19 \times 10^{-15}\ \text{mole P/cell,}$$

is about 14 times higher than the initial value, but three times less than maximum cellular-P levels reported by Kuenzler and Ketchum (1962) and indicates that these cells were still not saturated. In the other two species not more than half of each substrate was used. The uptake rate decreased noticeably with time in *Cricosphaera,* perhaps indicating that it is more quickly satiated.

The above experiments indicate that, instead of assimilating the whole ester, the algae hydrolyzed the ester extracellularly and took in the phosphate. One might expect the uptake of whole glycerol phosphate, sugar phosphate, or nucleoside phosphate molecules to proceed by different mechanisms and at different rates. However, the remarkable similarity in rates of accumulation of phosphorus from these three esters suggests that the rates were limited by a common mechanism. Alkaline phosphatases of other organisms frequently hydrolyze more than one phosphomonoester (Stadtman, 1961), sometimes at similar rates (*E. coli;* Torriani, 1960). Thus, the enzymatic hydrolysis rate might have limited the rate of phosphorus uptake from these three esters. On the other hand, if sufficient phosphatase were present, the phosphate assimilation rate would be the limiting factor. The phosphorus content of the *Phaeodactylum* inoculated into each of the three esters (Fig. 4) was 1.4×10^{-15} mole/cell. Kuenzler and Ketchum (1962) found the phosphate uptake rate of *Phaeodactylum* in an 8 μM solution to be of the order of 0.32×10^{-15} mole/cell·min. for two hours. These two values give an expected doubling time of cellular phosphorus of 4.4 minutes; cellular P actually doubled more quickly than this in the initial period (Fig. 4) when the sea water phosphate was being assimilated. The lower rate of P accumulation from the esters than from the sea water phosphate suggests that insufficient phosphatase was present to release phosphate as rapidly as the cells could accumulate it and, therefore, that the ester hydrolysis rate was the limiting factor. The experiments were done at about pH 8 although the phosphatases of these three species are more active at higher pH values (Table III). Here, however, we were interested in the capabilities of algae under approximately normal conditions rather than under conditions optimal for one enzyme but possibly damaging to the whole, living cell.

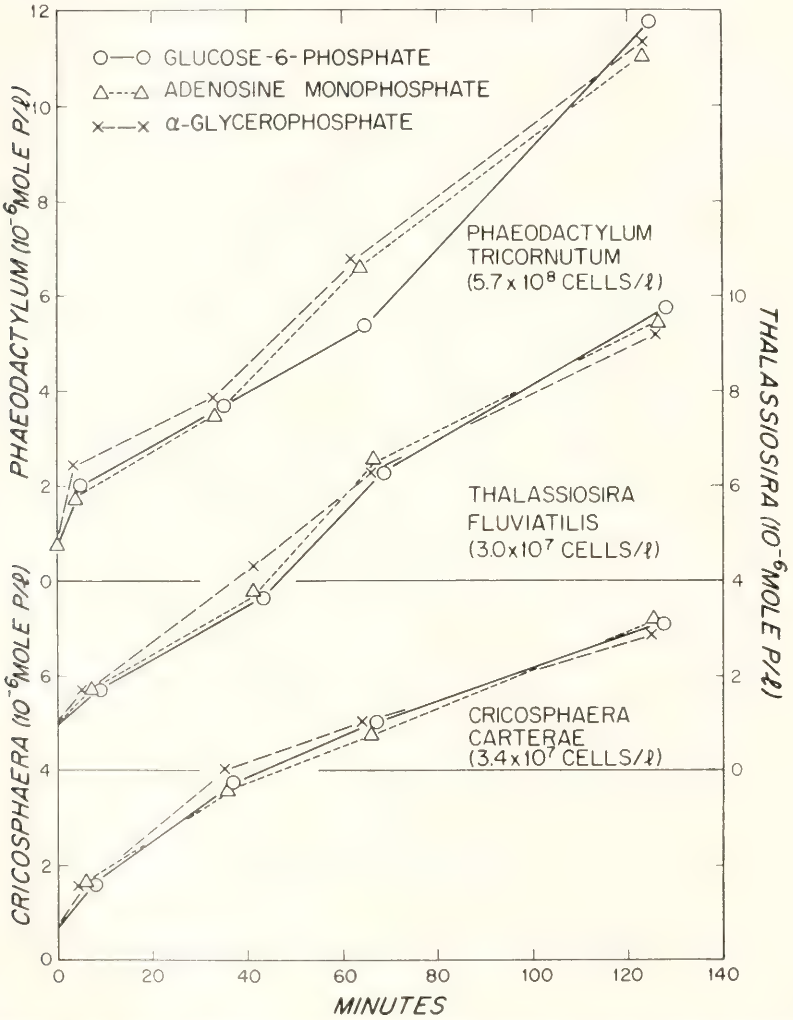

Figure 4. Increase in phosphorus content of three species of algae when exposed to three phosphate esters. The initial concentrations of G-6-P, AMP, and α-GP were 12–14, 22 and 14–16 μM P, respectively.

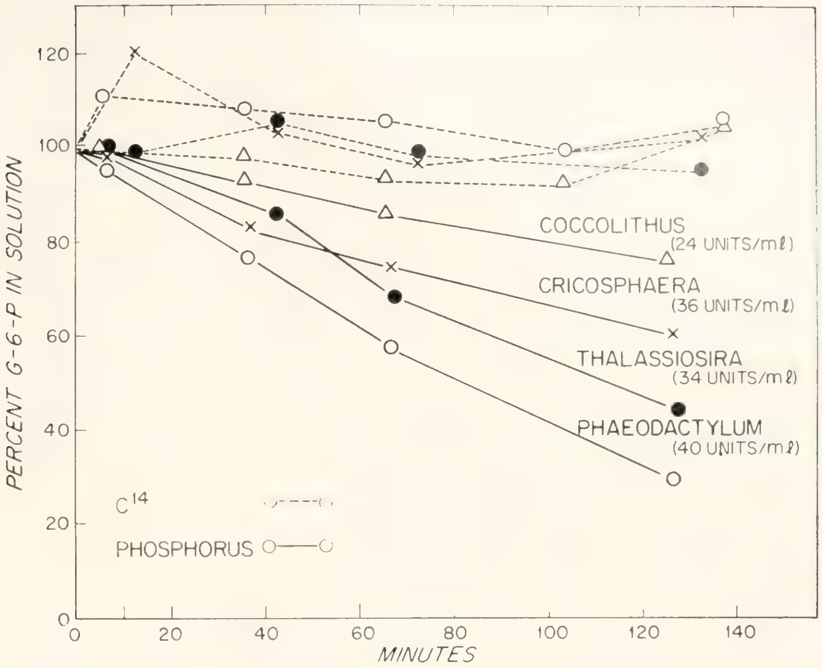

FIGURE 5. The hydrolysis of C^{14}-labeled glucose-6-phosphate by P-deficient *Coccolithus huxleyi, Cricosphaera carteri, Thalassiosira fluviatilis* and *Phaeodactylum tricornutum.* The concentration of the glucose moiety in the medium was determined by measuring C^{14} (dashed lines); the concentration of phosphorus remaining in solution (solid lines) was determined by the difference between the original concentration and the amount measured chemically in the cells. Enzyme activity was measured with NPP at the same time in other portions of each culture; results are shown in parentheses (Units/ml. of culture).

The following experiment showed that only the phosphate is assimilated and that the organic part of the ester remains in solution. When C^{14}-labeled G-6-P was mixed with four cultures of P-deficient algae, radioactive carbon remained in solution while the phosphate was assimilated by the cells (Fig. 5). (The variability in the C^{14} counts was probably caused by self-absorption and geometry variability.) The simplest explanation is that the phosphate was hydrolyzed extracellularly by phosphatase and taken in, whereas the glucose never entered the cell at all. This is substantiated by the general correspondence between the slopes representing the decline of phosphorus in the medium and the phosphatase activity as measured independently with *p*-nitrophenyl phosphate.

Location of the enzyme

The alkaline phosphatase of the two species studied more carefully, *Coccolithus huxleyi* and *Phaeodactylum tricornutum,* is firmly attached rather than being in solution in the cell. Repeated attempts on one or the other species, using sonication, explosive decompression, osmotic shock, freezing and thawing, heating, or treatment with lipase, cellulase, iso-butanol, and various salt and buffer solutions, failed to solubilize the enzyme.

Rothstein (1954) discussed several criteria that might indicate enzyme attachment to a cell surface rather than to an internal organelle, and we have applied some of these criteria to our own data. If the enzyme is located at the cell surface, disrupted cells should show no more activity than whole cells and the pH responses of disrupted and whole cells should be the same. When we sonicated, then centrifuged, cultures of *Coccolithus huxleyi* and *Chlamydomonas reinhardi,* all the enzyme activity was found in the pellet; additionally, the activity of the pellet was no more than that of whole, living cells. Experiments with six other species (Table IV) showed that little of the enzyme activity was in solution either before or after sonication. Here, too, except for *Cricosphaera,* there was no increase in enzyme activity after disruption of the cell structure. In *Coccolithus* the activity of each pH between 7.7 and 9.7 was approximately the same in sonicated as in intact cells. As already discussed, identical rates of assimilation of P from G-6-P, AMP, and α-GP more likely result from hydrolysis at the cell surface than from identical rates of transport of the whole molecules into the cells. Finally, the nitrophenol resulting from hydrolysis of NPP was found in solution, just as the C^{14} from labeled G-6-P stayed in solution (Fig. 6); these facts are more readily explained by extracellular hydrolysis of non-penetrating substrates than by immediate excretion of nitrophenol, labeled glucose, or labeled CO_2 following intracellular hydrolysis. Our evidence for cell-surface enzymatic activity in algae agrees with that of Brandes and Elston (1956), Talpasayi (1962), Galloway and Krause (1963), and Overbeck (1962) already mentioned in our introduction.

TABLE IV

Alkaline phosphatase (Units/ml. of culture) in cells and filtrate before and after sonication. The cells were sonicated at 0° C. in 5 N NaCl

	Whole culture		Sonicated culture		
	Cells	Filtrate	Cells		Filtrate after
			Before	After	
Phaeodactylum tricornutum	90.4	9.6	83	80	9.2
Cyclotella cryptica	50	0	52	38	0.1
Chaetoceros simplex	73	0	72	58	0.
Thalassiosira fluviatilis	64.4	1.6	74	57	0.24
Isochrysis galbana	130	0	103	70	0
Cricosphaera carterae	141	0	119	201	0

DISCUSSION

It appears that the alkaline phosphatases of marine algae may enable them to regenerate phosphate from organic sources and thus circumvent heterotrophic regeneration. In general, heterotrophs may be expected to hydrolyze phosphate esters in order to obtain the energy bound in the organic moiety, whereas autotrophs with abundant energy available from sunlight would only need the inorganic phosphate radical. There are similarities, however, between phosphatases of yeasts and bacteria and those of algae. Suomalainen *et al.* (1960) reported that both the acid and alkaline phosphatases of baker's yeast increased markedly with phosphorus starvation. From comparisons of enzyme activity of whole cells to that of dried, freeze-thawed, or cytolyzed cells, they concluded that the acid phosphatase was mostly at the cell surface and the alkaline phosphatase was mostly internal. In the bacterium, *Escherichia coli,* Torriani (1960) found that the acid phosphatase was always present (*i.e.,* constitutive), whereas alkaline phosphatase synthesis was repressed by phosphate. Malamy and Horecker (1961) removed the cell walls from *E. coli* with lysozyme and reported that these protoplasts lost most of their alkaline phosphatase activity. They suggested that the enzyme lies outside the cell membrane. Yeasts and bacteria, therefore, also have a means of hydrolyzing phosphate esters extracellularly, especially when P-deficient.

The ability to produce phosphatases that act on extracellular substrates may give some algae a competitive advantage over other species when phosphate in sea water is less abundant than dissolved organic phosphorus. It is probable that these phosphatases not only hydrolyze but also immediately transfer the phosphate to an acceptor in the cell. Such a transfer would improve the recovery efficiency and increase the competitive advantage even more. The components of the dissolved organic phosphorus pool in the sea are still completely unknown, but undoubtedly some of the compounds are phosphomonoesters. Kuenzler *et al.* (1963) reported that P-deficient *Phaeodactylum tricornutum* could rapidly obtain more phosphorus from natural sea water than was present as orthophosphate, and concluded that the difference came from dissolved organic phosphorus compounds.

We have tried to learn the characteristics of the phosphatases of whole algae and the resulting capabilities for the living cells. In this regard, experiments on enzyme activity at very high or very low pH (Fig. 3) are not very meaningful because such conditions are abnormal in the sea and often lethal in cultures. Sea water usually has a pH of 8 or higher where active photosynthesis is occurring, and it seems significant that the phosphatases synthesized can hydrolyze esters at sea water pH. Constitutive internal phosphatases can be expected to have optima suited to particular pH values of various sites within the cell.

It is possible that the ratio of phosphatase to cell volume, protein, or chlorophyll in natural phytoplankton populations can give direct information about their nutrient status. The fact that phosphate is undetectable in sea water does not prove that the natural phytoplankton population is phosphorus-deficient. The limit of chemical detectability is not yet as low as the limit of assimilability by algae (Kuenzler and Ketchum, 1962). From Tables II and III we would guess that samples of phytoplankton dominated by chrysophytes and diatoms are phosphorus-deficient if the alkaline phosphatase activity is greater than 10×10^{-8} Units/μ^3 of cells, *i.e.,* two days' production of this enzyme at a rate of 5×10^{-8} Units/$\mu^3 \cdot$day. We have been able to detect alkaline phosphatase in the particulate matter of some samples of natural sea water. More analyses must be done, however, before we can draw conclusions about the phosphate nutrition of the plankton from enzyme measurements.

SUMMARY

1. Phosphate-repressible alkaline phosphatases were found in axenic cultures of marine algae, especially Chrysophyceae and Bacillariophyceae. Enzyme synthesis

started when the algae became phosphorus-deficient and stopped if phosphate was restored to the medium.

2. Maximal enzyme activity was usually above pH 9, but significant activity was also present at ordinary sea water pH, approximately pH 8.

3. The phosphatases enabled algae to split glucose-6-phosphate; the phosphate was quickly assimilated but the glucose moiety remained in the medium. Algae took phosphorus from adenosine monophosphate and α-glycerophosphate at the same rate as from glucose-6-phosphate.

4. The phosphatases appear to be firmly bound near the cell surface. They may enable deficient algae to regenerate phosphate from soluble organic phosphorus compounds present in natural sea water.

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STABILIZATION OF THE VISUAL FIELD[1]

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Most animals can make no adjustment in the orientation of their principal visual organs in relation to the body. Those that can move their eyes separately are the vertebrates, virtually all of the cephalopod mollusks, many crustaceans, and the comparatively few kinds of insects that have a mobile head. A remarkable number of these animals use this ability in a paradoxical way. They move their eyes *in relation to the body* to prevent their eyes from moving *in space.* Involuntary movements of their eyes tend to stabilize their visual field.

The anatomical basis for these adjustment is well known among vertebrates, which uniformly possess a conical array of four rectus muscles, and two oblique muscles in the orbit of each eye. Involuntary oscillatory adjustments (nystagmus) of the eye, chiefly through contractions of the external and the internal rectus muscles, have been noted for many years in man and some other vertebrates, elicited by visual stimuli. The obliques provide a correcting system that smooths out the horizontal and vertical sweeps produced by the antagonistic rectus muscles. They also produce "wheel movements" (Raddrehungen, Augenrollungen, cyclorotations) that confer on the organ a third degree of freedom. With its obliques, an animal may also be able to correct for skewness in its visual field. Involuntary responses of this kind follow stimulation of sensory centers in the muscles of the human neck (Nagel, 1896; Zoth, 1905), and in the inner ears of several other vertebrates (Benjamins, 1918, 1920; Magnus and de Kleyn, 1921). These adjustments based upon proprioceptive and gravitational cues show a latency in milliseconds, and commonly depend upon the obliques acting alone.

Voluntary use of the rectus muscles in opposing pairs is found in some but not all vertebrates, and serves to keep centered in the visual field any object that moves. This following is usually jerky, with momentary pauses for fixation. Only these voluntary movements are discussed under ocular motility in *The Vertebrate Eye and Its Adaptive Radiation* (1942) by the late Gordon L. Walls, and in *Les Yeux et la Vision des Vertébrés* (1943) by the outstanding French ophthalmologist A.-J.-F. Rochon-Duvigneaud. The latter divided reptiles into a category with immobile eyes (the snakes, geckos, and crocodilians) and another with mobile eyes (most lizards and chelonians). The true rarity of voluntary movements presumably led Sir Stewart Duke-Elder in his comprehensive volume, *The Eye in Evolution* (1958), to comment that most birds have fixed eyes and compensate for this fixity by bending their necks to follow objects of interest.

Benjamins (1918) rotated a live perch about a transverse axis and recorded the wheel movements of its eyes (Fig. 1A). When the anterior end of the fish was raised, to a position matching that assumed by the animal while feeding at the surface, compensatory rotation of its eye maintained the horizontality of the horizon to about 10 degrees, and then failed progressively to maintain correspondence as the angle between the longitudinal axis of the body and the horizontal was increased (Fig. 1B). A maximum rotation of 28 degrees in this direction was reached when the body reached a 70-degree angle with the horizontal. Stability of the visual field was never as good when the anterior end of the fish was depressed, as it would be while the animal feeds from the bottom. A maximum rotation of the eyes was reached at 35 degrees when the body made an 80-degree angle with the horizontal. Curiously, the cyclorotational correction vanished when the fish was inverted and its longitudinal axis again lay in the horizontal plane. These involuntary responses appear to depend entirely upon stimulation of the maculae of the inner ear.

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[1] Presented before the Fourth International Congress on Photobiology, at Oxford, England, July 27, 1964.

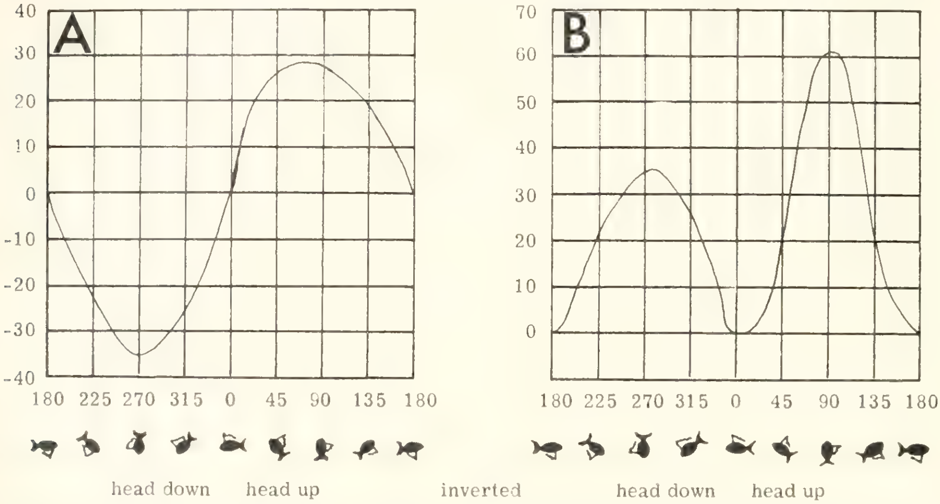

FIGURE 1. When a European perch is rotated slowly about a transverse axis, its eyes exhibit compensatory rotational adjustments with respect to the body (*A*), but fail to hold the visual field level (*B*) except within about 8 degrees with head raised, about 5 degrees with head lowered, and about 1 degree with the body completely inverted. (Replotted from data of Benjamins, 1918.)

Magnus and de Kleyn (1921) found remarkable excursions of the eyes in European rabbits, according to the static position of the inner ears. It made no difference whether the whole animal was tilted or only its head was turned. Compensatory rotations of its eyes tended to stabilize the visual field within a degree or less over a range of head positions nearly 100 degrees in any direction. Beyond this limit, the rabbit's eyes tended to remain in their position of maximum excursion, and the animal showed a lessened response to visual stimuli.

Involuntary cyclorotation of the eyes in a reptile came to public notice through some observations of the copperhead snake (a pit viper of America) made by Professor D. F. Munro of Kansas State College and communicated by him to Albert G. Ingalls (*Scientific American,* March, 1954). Munro had noted that the vertical slit pupils of this snake remain vertical even when the reptile's head is raised at an angle of 65 degrees, or lowered a like amount. He reported further that just before dozing, the snake let the anterior pole of each eye rotate ventrad by about 10 degrees. When the snake was actually asleep, its pupils again took on a vertical orientation, but with the whole eye rotated until only the top of the pupil was visible through the corneal scale (Fig. 2).

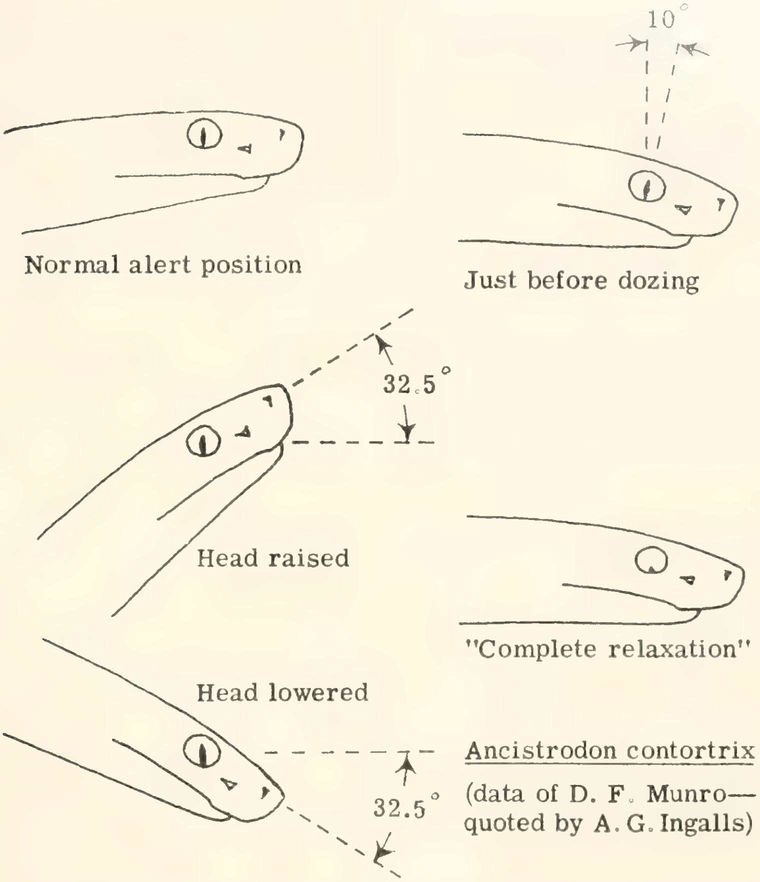
10°
Normal alert position
Just before dozing
32.5°
Head raised
"Complete relaxation"
Head lowered
32.5°
Ancistrodon contortrix
(data of D. F. Munro—
quoted by A. G. Ingalls)

FIGURE 2. Rotational movements of the eye in the copperhead snake. (Data of D. F. Munro, prepared by permission from an illustration. Copyright © 1954 by Scientific American, Inc. All rights reserved.)

In a subsequent issue of the same magazine (November, 1954), Ingalls published further information on cyclorotation, with observations made by Henri Morgenroth, a consulting engineer of Santa Barbara, California. The new data offered by Morgenroth related to a young turtle and a goldfish, and drew forth an additional comment from Professor Munro to the effect that stabilization of the visual field by cyclorotation is found also in snakes that do not strike at prey. One with a vertical oval pupil (unidentified) was cited as showing stability of eye orientation with respect to the field over a range of head position from 27.5 degrees below to 27.5 degrees above the horizontal.

For the eye of the pigeon, Whitteridge (1956) found a rotational correction amounting to about 10 degrees for head positions with the beak raised or lowered beyond the normal orientation. He did not establish whether the adjustment was a response to stimuli detected in the inner ears or in neck muscles. In man, the neck receptors alone call forth the response, and no stabilization of the visual field is gained if the whole trunk and head are inclined together to right or left.

Analogous mechanisms can be recognized among several types of invertebrates. The slit pupil of the European *Octopus vulgaris* has long been seen to remain horizontal despite movements of the body over irregular obstacles (Magnus, 1902; Muskens, 1904; van Weel and Thor, 1936). These responses are obliterated if the statocysts are destroyed or the nerves from them are cut (Young, 1960). No measurements of the maximum rotational adjustments possible seem to have been published for this cephalopod.

Compensatory movements of the eyes have been observed also in crustaceans. When the mantis shrimp, *Squilla,* crawls up over an obstacle, it allows its eyestalks to droop (Demoll, 1909). The rotations of the almost spherical median compound eye of *Daphnia,* under rather jerky control of four oculomotor muscles, are a counterpart on a smaller scale (Ewald, 1914).

Since no systematic account of mechanisms providing stability to the visual field has yet appeared, we decided to collect together the scattered observations (and measurements) from the literature and to fill in gaps as opportunities allowed.

MATERIALS AND METHODS

Where possible, we have obtained photographic records of the extent of compensatory cyclorotation in a variety of living invertebrates and vertebrates (Table I). In some instances, as with the sand shark, *Carcharias,* it was inadvisable to molest the animal for the sake of discovering the fullest extent of possible compensation by displacing its body from its normal relationship to the horizontal plane. We photographed the shark at those times when it chose to pass the portholes of a large oceanarium tank, while it was swimming in a strongly nose-up or nose-down position. The values cited in the table for the shark are taken from 16-mm. motion pictures. For measurement we selected those frames in which the lens of the camera appeared to be facing the shark along the transverse axis from eye to eye. Other values cited in the table represent the maximum excursion noted. In all instances from our own studies, the maximum was reached before the animal's body was rotated 90 degrees about a transverse axis from its normal attitude with respect to the horizontal plane. We did not explore for any animal its rotational responses to body attitudes more than 90 degrees away from normal, since such extremes seem improbable under natural conditions. We may thus have overlooked counterparts of the phenomenon reported for the tadpole of the frog, *Phyllomedusa,* in which the eye locked in its normal orientation when the animal was turned on its back (Quesnel, 1956).

RESULTS

Vertebrates

Among the animals tested, only the spectacled caiman showed oscillatory rotational adjustments (a "cyclonystagmus") for several minutes whenever held with

TABLE I

Cyclorotational compensatory adjustments retain an essentially stable visual field to the limits shown, except in Perca, for lateral bending of the neck (in Homo) or for elevating (+) or depressing (−) the anterior end of the body. Reference points for detecting cyclorotation can be found in pigment flecks on the iris in Homo, Oryctolagus, Columba, Perca, and Cyprinus; in the vertical slit pupil of Caiman, Ancistrodon, Constrictor, and Hemidactylus; in a horizontal black stripe through sclera and iris in Pseudemys, Pseudotriton, and Necturus; in a vertical oval pupil in Bufo, Rana, and Carcharias; in a nick in the ventral edge of the circular pupil in Phyllomedusa; and in a horizontal slit pupil in Sepioteuthis

Response by class order	Organism genus and species	Degrees		Previously reported by
		+	−	
A. Rotation of the eye in its orbit				
Mammalia—Primates	*Homo sapiens*	8.6	8.6	Nagel, 1896; Zoth, 1905; Merton, 1956
Mammalia—Lagomorpha	*Oryctolagus cuniculus*	100	100	Magnus & de Klejn, 1921
Aves—Columbiformes	*Columba livia*	10	10	Whitteridge, 1956
Reptilia—Crocodilia	*Caiman sclerops*	10	45	
Reptilia—Squamata	*Ancistrodon contortrix*	32.5	32.5	Ingalls & Munro, 1954
	Constrictor constrictor	30	30	
	Hemidactylus mabouia	15	20	
Reptilia—Chelonia	*Pseudemys scripta*	70	10	Ingalls & Morgenroth, 1954
Amphibia—Diplasiocoela	*Bufo americanus*	5	0	
	Rana pipiens	0	0	
	Phyllomedusa burmeisteri (tadpole)	62.5	62.5	Quesnel, 1956
Amphibia—Mutabilia	*Pseudotriton ruber*	20	15	
Amphibia—Proteida	*Necturus maculosus*	15	5	
Osteichthyes—Percomorphi	*Perca fluviatilis*	28	35	Benjamins, 1918
Osteichthyes—Ostariophysi	*Cyprinus carpio*	60	60	Ingalls & Morgenroth, 1954
Chondrichthyes—Selachii	*Carcharias taurus*	20	20	[estimated]
Cephalopoda—Dibranchia	*Sepioteuthis sepioides*	20	20	
B. Bending head with eyes at neck				
Insecta—Odonata	*Anax junius*	8	8	
C. Bending eyestalks				
Crustacea—Decapoda				
by lateral adjustments	*Carcinides maenas*	20	20	Schöne, 1954
	Ocypode albicans	30	30	
	Podophthalmus vigil	65	65	
by vertical adjustments	*Astacus fluviatilis*	12	23	Kühn, 1919
	Homarus americanus	5	0	
D. Bending ocular plate and eyestalks (by vertical adjustments)				
Crustacea—Stomatopoda	*Squilla mantis*	80		Demoll, 1909
	Gonodactylus oerstedi	60	5	
E. Rotating median fused compound eye				
Crustacea—Cladocera	*Daphnia pulex*	15	15	Ewald, 1914

its head raised more than 10 degrees or lowered more than 50 degrees. Possibly when this animal is allowed to move freely, it seldom assumes these attitudes.

Cephalopods

The same limits for cyclorotation were noted in the squid, *Sepioteuthis,* when specimens were restrained by hand in a marine aquarium and when free to move undisturbed. Unrestrained squids in captivity often assume attitudes approaching the extremes for which their cyclorotational compensation seems adequate. Less extreme positions were observed under natural conditions over the reef, upon those occasions when small schools of squid passed close to us as we watched horizontally through glass face plates while submerged.

Insects

The value shown in the table for the dragonfly, *Anax,* with its anterior end depressed is close to the limit imposed by contact between head and thorax. Possibly this attitude is unusual for the insect, whether at rest or in flight. Compensatory movements that might stabilize the visual field were looked for but never found in the introduced mantis, *Paratenodera sinensis.* Mantises appear to look about themselves alertly, making wide use of a flexible neck. They also cling in many positions while waiting for prey. Females stand head downward while producing an egg mass.

Crustaceans

Among decapod and stomatopod crustaceans, the eye movements that tend to stabilize the visual field are of different types according to the normal manner of progression shown by the animal. For crabs which run from side to side, the pertinent movements of the eyestalks are those shown when the crab goes sidewise up or down a slope. For crayfishes, lobsters, and stomatopods, all of which progress forward when hunting and backward when escaping, the comparable changes in attitude of the eyes are those that match elevation or depression of the anterior end (the head) with respect to the abdomen.

Among crabs, the tendency is to maintain vertical the whole eyestalk or that part bearing the compound eye. Among crayfishes, lobsters, and stomatopods, the eyestalk is more commonly held almost horizontal. In each species examined, these orientations of the eyes are maintained well during changes in body position within the limits cited in Table I. Gravity is presumed to determine the orientation, since it is maintained in complete darkness.

In the cladoceran, *Daphnia,* by contrast, gravity appears to have no role in responses involving the oculomotor muscles. These muscles respond only when the pattern of illumination on the ommatidial cluster meets certain specifications. No eye movements are elicited under uniform red light to which *Daphnia* is insensitive (Ewald, 1914), or when unpolarized light reaches the animal from above the water while *Daphnia* is in its upright swimming position. If either the light comes from a different angle or the *Daphnia* is differently oriented, ommatidia other than the "usual" ones appear to receive greater stimulation. The eye is then rotated, and swimming movements show adjustments that bring the animal into the orientation in which no further compensatory rotation is called for.

Discussion

Mechanisms that tend to stabilize the visual field while the head or body is displaced from its commonest orientation in space all have one adaptive value. They aid the eyes in bringing to the brain a pattern it may recognize, as the basis for a response that may have survival value to the animal.

Among non-primates, where reactions to specific patterns in the visual field appear to be innate, this matching of information from the eyes with the central identification system would seem particularly important (Morgenroth, as quoted by Ingalls, 1954). Even where conditioned responses develop readily, the orientation of the pattern with respect to the horizontal plane may be important. Supposedly this is the basis for the ability shown by a captive *Octopus* to learn that a reward lay behind a horizontal rectangle but punishment behind a vertical one (Boycott, 1954). It retained the ability to discriminate so long as its statocysts and the nerves connecting them to the brain were intact. Ability to make compensatory cyclorotations of the eyes in relation to altered orientation of the body also vanished permanently when the statocysts were destroyed or their nerve connections cut.

People, too, learn to identify patterns quickly in one orientation and sequence, and have difficulty transferring this learning to a different orientation or a different sequence. For any person trained in a Western alphabet, the sequence of left-to-right seems more important than inversion. If forced to read a printed page from a mirror image or upside down, we decipher the familiar words slowly and laboriously. A printer avoids this difficulty when he sets type by hand, working from left to right with the top of all characters toward him. In this orientation he can proof each line of type quickly, recognizing every letter inverted but in its normal sequence.

Similarly, the lettering on the backbone of a book is read far more quickly if we can turn our head 90 degrees and see the letters in customary relationship, side-by-side, than if the letters are placed one above the next and must be read from top to bottom with our head in its upright position. Visual acuity falls off rapidly if our scanning movements must follow unusual directions (Merton, 1956).

For insects, the orientation of a pattern in relation to the horizontal plane may be far less important than other sensory cues derived from the relative attitudes of the head and body. When Miss Mathilde Hertz showed (1929–1934) that the honeybee readily confuses patterns having the same general ratio of perimeter to area, biologists were somewhat surprised. But now it is clear that this insect discriminates among the patterns it encounters with no need to turn its head, and gains from relative movements of head, thorax, and abdomen a multitude of cues important in maintaining stability in flight (Lindauer and Nedel, 1959). In this respect it parallels the sensory system in a dragonfly (Mittelstadt, 1950) whose delicately-held massive head maintains its orientation in space through inertia despite vertical currents of air that tilt the outstretched wings. Although a dragonfly can bend its head as much as 25 degrees to reach with its mouth for insects caught among its forward-reaching legs, it shows little of the compensatory rotation of the head that the flexible neck makes possible. While clinging to a vertical reed, the dragonfly ordinarily holds its head much as it does in flight, with the sensory pads on the sides of its neck within grazing distance of skeletal projections on the head and thorax. Reflexes mediated through these pads are called forth whenever the head and thorax are out of line. They normally lead to changes in wing movements that bring the dragonfly back to a straight and level flight path.

Among crustaceans with stalked eyes, analysis is complicated by the fact that the eyestalk fundamentally consists of two segments. The distal one is the "calathus" of Young (1959), and bears the compound eye. The proximal segment, next to the cephalothorax, is greatly reduced in most decapods. But in a few the proximal segment is far longer than the distal one and the crab gains from flexibility between the two.

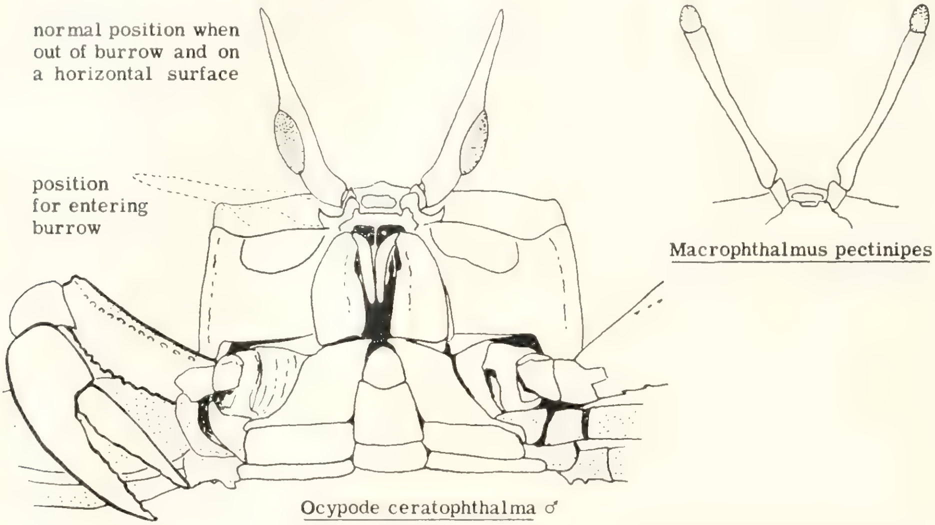
normal position when out of burrow and on a horizontal surface
position for entering burrow
Macrophthalmus pectinipes
Ocypode ceratophthalma ♂

FIGURE 3. Lateral adjustments of the eyestalk are used to stabilize the visual field in the ghost crabs, *Ocypode ceratophthalma* and *Macrophthalmus pectinipes.* In both, the proximal segment of the eyestalk is greatly reduced, and movements involve only the elongated distal segment.

The ghost crabs, *Ocypode ceratophthalma* and *Macrophthalmus pectinipes* (Fig. 3), show special development of the distal segment. Like *Ocypode albicans,* these terrestrial crabs tend to stabilize their visual field over a wide range of body positions. To do so they use the same muscles that serve to raise and lower the eyestalks into the protective groove across the anterior edge of the carapace. When a ghost crab begins the downward slant into its burrow in the beach, it quickly lowers the eyestalk on the advancing side and then the eye on the opposite side as it enters the hole. Upon return to the surface the crab raises its eyestalks vertically at once, then drops them part way into the groove while quickly wiping them clear of sand with the flexible tips of the third maxillipeds. Orientation of the eyestalks appears to depend upon specialized sensory hairs in the statocyst which is present in the proximal segment of the antennules (Dijkgraaf, 1955, 1956; Schöne, 1951, 1954, 1959; Cohen, 1960). Statoliths are lacking (Bethe, 1897).

A similar mechanism appears to control the distal segment of the eyestalks in *Podophthalmus* and *Euphylax,* which possess statocysts with specialized hairs but no statoliths. In these marine crabs of shallow coastal water, the proximal segment of the eyestalk is greatly elongated, whereas the distal segment is scarcely larger than the compound eye (Balss, 1940). According to Mr. Bryant Sather (personal communication), *Podophthalmus vigil* of Hawaii holds its proximal segments at 45 to 50 degrees above the horizontal by day, and about 10 degrees at night (Fig. 4). When feeding in silt that envelops the body completely, *Podophthalmus* may raise its proximal segments to a still greater angle, perhaps 65 degrees. Often this suffices to keep its compound eyes in clear water above the silt, where vision is possible. Both at night and when the proximal segments are elevated into daytime position, the distal segments are kept vertical. Only when the crab is lifted out of the water or its eyestalks are molested is the distal segment lowered into the protective groove across the anterior edge of the broad carapace. Thus, the elongated proximal segments serve to raise the eyes high above the body of this silt-dwelling crab, while muscular adjustments of the short distal segment stabilize the visual field.

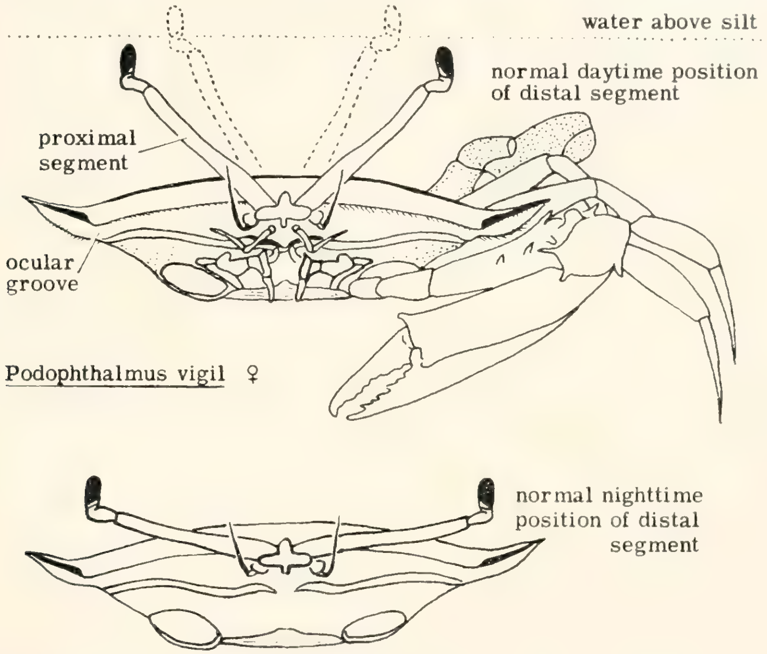
water above silt
normal daytime position of distal segment
proximal segment
ocular groove
Podophthalmus vigil ♀
normal nighttime position of distal segment

FIGURE 4. The Hawaiian crab, *Podophthalmus vigil,* maintains the verticality of the distal segment of each eyestalk while moving about with the proximal segments raised by day or partly lowered at night, and while moving sidewise over an inclined surface. (Data from Sather, 1962.)

Sand grains, serving as statoliths in the antennular statocysts of crayfishes and lobsters, appear necessary in the sensory system that permits these animals to right themselves and make compensatory adjustments of their eyestalks to match rotational displacements of the body (Kuhn, 1919; Schöne, 1954). But when the lobster, at least, walks over an irregular bottom and raises its anterior end to surmount some obstacle, its eyestalks droop only slightly. No compensatory movements of the eyes are apparent when the lobster descends a slope.

Stomatopod crustaceans lack statocysts altogether (Demoll, 1909; Balss, 1938), and make most of their compensatory adjustments by lowering the eyestalks in relation to the ocular plate, which is a hinged forward portion of the head region. These adjustments, which are conspicuous while the animal crawls up over some obstacle, occur at the same time that the eyestalks are performing scanning movements (Milne and Milne, 1961).

Organization of the compound eye in the cladoceran, *Daphnia,* is on an utterly different plan. The 22 ommatidia are radially arranged around a median mass that arises by fusion of a pair of embryonic rudiments. With so simple a compound eye, *Daphnia* can gain only the crudest resolution from its field of view. The structure is at least two orders of magnitude simpler than any of the other animals discussed above. Seemingly the compensatory movements of the eye, which the four oculomotor muscles control, are unrelated to any feature of the field of view other than the most intensely illuminated part, and the principal plane of polarization if this is detectable.

We are particularly grateful to Mr. Bryant Sather, Department of Zoology, University of Hawaii, for his notes on *Podophthalmus* and specimens of this remarkable crab. Our own observations on *Caiman* and *Constrictor* were made at the University of the West Indies, Mona, St. Andrews, Jamaica, B.W.I., through the courtesy of Mr. Garth Underwood; on *Hemidactylus* and *Sepioteuthis* at the Bellairs Marine Laboratory of McGill University, Barbados, B.W.I., with assistance from Dr. John Lewis; on *Carcharias* at Marine Studios, Marineland, Florida; on *Ocypode* and *Gonodactylus* at the Lerner Marine Laboratory of the American Museum of Natural History, Bimini, Bahamas, B.W.I.; on the musculature of *Squilla* from large specimens generously furnished by Dr. Carl N. Shuster, Jr., now of the Northeast Shellfish Sanitation Research Center, Davisville, R. I.; and on *Bufo, Rana, Pseudotriton, Necturus,* and *Homarus* at the University of New Hampshire. Travel costs were defrayed in part by research grants from the Explorers Club, the Society of the Sigma Xi, and the Central University Research Fund of the University of New Hampshire.

SUMMARY

1. Stabilization of the visual field through cyclorotational movements of the eyes has been recorded in representatives of all major groups of vertebrates and in cephalopod mollusks possessing camera-style eyes. New data are offered on *Caiman, Constrictor, Hemidactylus, Bufo, Rana, Pseudotriton, Necturus, Carcharias,* and *Sepioteuthis.*

2. Comparable adjustments in the movable stalked eyes of crustaceans have been noted in both brachyuran and macruran decapods and in stomatopods. New data are offered on *Ocypode, Podophthalmus, Homarus,* and *Gonodactylus.* The silt-dwelling crab, *Podophthalmus,* represents an extreme, in which the elongated

proximal segment of the eyestalk raises the terminal segment high above the body, while the terminal segment is stabilized in relation to gravity and usually extends above the silt into clear water.

3. Movements of the head in insects that have a flexible neck appear to relate less to stabilization of the visual field than to feeding, cleaning the legs and body, or recovery of normal flight attitude after displacement by air currents. Orientation of patterns in space seems to have less significance than other parameters of important patterns. Some compensatory movements of the head are reported for dragonflies (insect order Odonata) but not for mantises (order Orthoptera).

4. Oculomotor activity of the median compound eye in the cladoceran *Daphnia* shows no definite relationship to gravity. Its responses to light represent a much lower order of reaction to the environment than is noted in malacostracan crustaceans with mobile compound eyes.

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ECHOLOCATION OF FLYING INSECTS BY THE BAT, CHILONYCTERIS PSILOTIS

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Among the many aspects of acoustic orientation in bats that have been analyzed, studies of sonar design as it varies during insect pursuits and obstacle avoidance have been limited to a few genera, principally of vespertilionid bats (Griffin, 1953, 1958; Grinnell and Griffin, 1958, Griffin, Webster, and Michael, 1960; Cahlander, McCue, and Webster, 1964; Webster, 1963; Griffin, Friend, and Webster, 1964). Griffin (1962) has also reported briefly on pursuit behavior in *Noctilio* (Noctilionidae) and *Rhinolophus* (Rhinolophidae). Recently we have observed echolocation during insect pursuits in *Pteronotus* (Novick, 1936b) and in *Chilonycteris parnellii* (Novick and Vaisnys, 1964), closely related bats of the subfamily Chilonycterinae (Phyllostomatidae).

Systematic alterations in pulse duration and interpulse interval (or pulse repetition rate) have been described in *Pteronotus* and *C. parnellii* which imply a dependence, for detection of insect prey, on pulse-echo overlap at the bat's ear and which further imply the value to these two bats of adjusting pulse duration relative to target distance so as to give fairly constant pulse-echo overlap sequences during insect pursuits. Such apparent utility of pulse-echo overlap encourages consideration of the possibility of the bat's using a variety of analytical methods which have been briefly discussed by Pye (1960, 1961a, 1961b), Kay (1961, 1962), and Novick and Vaisnys (1964). The systematic variations in pulse duration have also implied measurement of target distance since pulse duration has, during a part of each pursuit, varied directly proportionally with bat-target separation. Variations in interpulse interval may be related to reaction time in the central nervous system and/or conservation of output.

The sonar design of *C. parnellii* differs strikingly from that of *Pteronotus,* as, indeed, it does from *C. psilotis* (Griffin and Novick, 1955; Novick, 1963a). These differences in design, principally pulse duration and intensity, increased our interest in comparing the behavior of the members of this closely related group of bats, in the hope of unveiling some of the functions of the sound parameters used.

The orientation sounds of one individual *Chilonycteris psilotis* Dobson (Phyllostomatidae) (Hall and Kelson, 1959) were recorded while the bat flew around a laboratory flight room and pursued and apparently captured common fruit flies, *Drosophila* sp. The orientation of *C. personata* has previously been studied by Griffin and Novick (1955) and Novick (1963a). This bat is either the same as *C. psilotis* or a very close relative. These bats are delicate in captivity. Of several individuals captured in a cave at Lake Tequesquitengo, Morelos, Mexico, only one survived to pursue fruit flies in New Haven and this one did so for less than a week. I am grateful to Dr. O'D. W. Henson, Jr. and Mr. R. M. Holt for capturing and maintaining this bat, to Dr. B. Villa-R. for his advice and aid, to the Institute of Biology of the National University of Mexico for the use of their facilities and to the Mexican government for permission to capture and transport this bat. This work was supported in part by the National Institute of Mental Health and by the Air Force Office of Scientific Research.

The bat's orientation sounds were recorded with a custom-made Granath microphone and a Precision Instrument Pi-202 tape recorder. Nine hunting sequences were chosen for analysis because of their high signal-to-noise ratio and completeness. These were analyzed chiefly from filmed oscillograph tracings. A Kay Electric Co. sound spectrograph was also used.

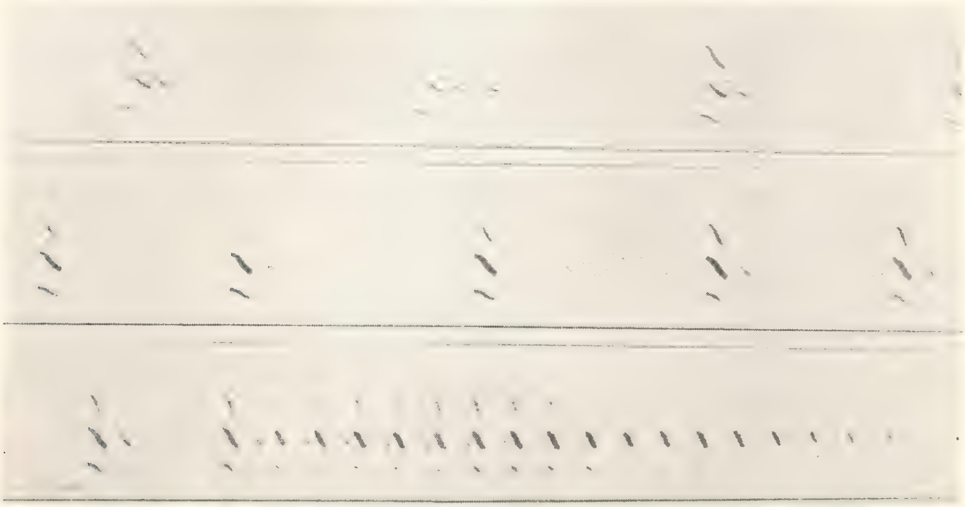

FIGURE 1. A sound spectrogram of the orientation pulses of *C. psilotis* during a fruit fly pursuit. The three strips are sequential with the last pulse of each repeated at the beginning of the next strip to increase clarity. Frequency is shown vertically; time horizontally. Echoes of many of the pulses appear as fainter duplicates a few milliseconds later. Note the three frequency components, the change in frequency drop during the pursuit, and the systematic changes in pulse duration and interpulse interval.

The present analysis deals chiefly with the significance of variations in pulse duration, interpulse interval and/or pulse repetition rate, and frequency pattern as seen in these records of insect pursuits, and with calculated pulse-echo overlaps. As in the vespertilionids (Griffin, Webster, and Michael, 1960), *Pteronotus* (Novick, 1963b), and *C. parnellii* (Novick and Vaisnys, 1964), these pursuits may be subdivided into search, approach, and terminal phases (Figure 1).

The search phase cannot yet be distinguished, in the case of any bat, from other normal, cruising orientation sound sequences. Presumably, during this phase, the bat has no knowledge of the insect it is yet to pursue or, at least, takes no observed behavioral notice of such. Clearly, a cruising bat is receiving information from a complicated laboratory environment (either as echoes or lack of echoes or as echoes of uninteresting timing) and the bat must be adjusting its flight so as to avoid walls and ceiling, etc., but we have not yet recognized patterns of behavior in cruising flight other than an apparent varying regularity. Two such long, cruising sequences, recorded at high amplitude and lacking patterns suggesting insect pursuit, have been analyzed. These lasted 770 and 870 msec., respectively. Pulse durations of 2.9 to 4.8 msec. occurred, with a mean of 4.0 msec. Interpulse intervals (the silent period between pulses) varied from 44 to 84 msec. with a mean of 56 msec.; almost all lay between 44 and 65 msec. The repetition rate was about 17–18 pulses per second in both series. Thus, searching flight in *C. psilotis* seems to consist of a sequence of pulses about 4 msec. in duration, spaced about 60 msec. apart.

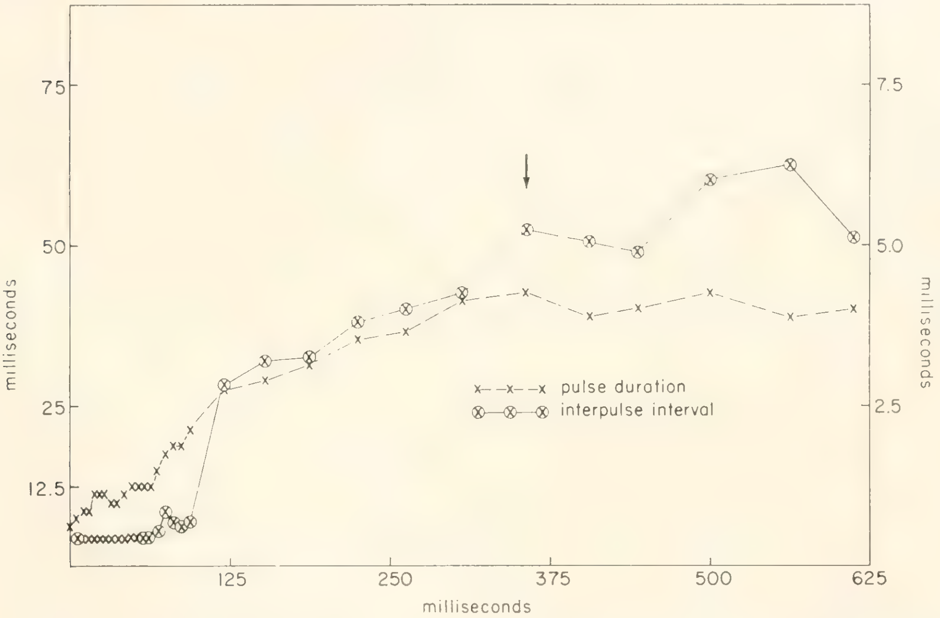

FIGURE 2. An example of a fruit fly pursuit by *C. psilotis*. Pulse duration and interpulse interval are plotted against milliseconds before the end of the pursuit. One msec. = 1.73 mm. (see text). The arrow indicates the first calculated pulse-echo overlap. Note that in Figures 2 through 5, time reads from right to left (larger numbers represent earlier events).

The approach phase begins, by definition, in these analyses, at the point at which we first recognize that the bat is committed to a complete pursuit. In *Pteronotus* (Novick, 1963b), this point was recognized by the first obvious shortening of the interpulse interval. Systematic changes in pulse duration also occurred, but their initiation point was harder to pinpoint. In *C. parnellii* (Novick and Vaisnys, 1964), the beginning of the approach phase is signaled by an increase in pulse duration while changes in interpulse interval occur gradually and are initially hard to identify. In *C. psilotis*, the beginning of the approach phase is marked by shortening of both pulse duration and interpulse interval (Figs. 1 and 2). During the approach phase, the bat apparently clarifies the position, nature, and velocity of the target and sets its own flight path for interception.

When plotted against time, close to linear shortening of the pulse duration and interpulse interval characterize this phase. It terminates sharply in a transition to a series of evenly spaced, short pulses of high repetition rate called the terminal phase (Figs. 1 and 2). In the terminal phase, the bat presumably closes in on, captures, and begins to eat the insect. Following the terminal phase there is a long silent period and then a return to searching sound production.

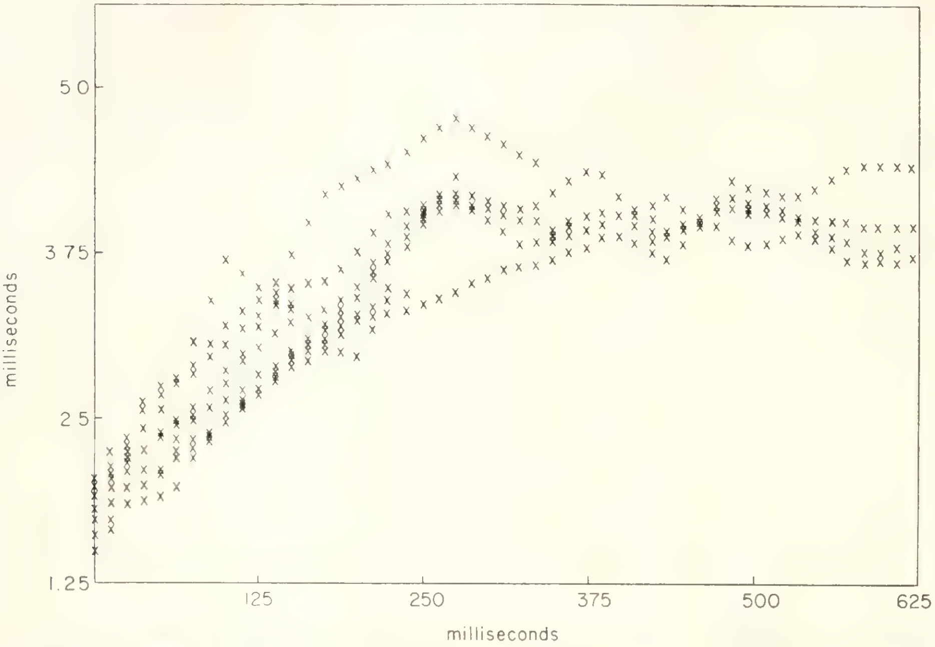

FIGURE 3. Nine fruit fly pursuits by *C. psilotis.* The pursuits have been synchronized by assuming equality of situation at the beginning of the terminal phase which is shown here as zero. Pulse durations preceding the beginning of the terminal phase are plotted against time during the pursuit. Intermediate interpolated values (between pulses) are plotted for equal time intervals. Note the change in the distribution of points at about 250 msec. before the beginning of the terminal phase. One msec. = 1.73 mm.

The nine best pursuits recognizably occupy 190 to more than 350 msec., measured from the pulse preceding the first recognized decrease in pulse duration. If the pulse preceding the first recognized decrease in interpulse interval is used instead as the index, the same pursuits last 210 to 360 mesc. Durations, by both criteria, of about 300 msec. are the most common. The terminal phase, identified by short interpulse intervals, occupies 28 to 94 msec. of this time. Excluding one terminal phase which is so short that it seems possible that the target escaped, the range is 50 to 94 msec. Thus, the approach phase *per se* ranges from about 150 to 290 msec. The measurements are objective except for the need to choose inflection points—the beginnings of the approach and terminal phases.

If one plots all of the pursuits (pulse duration and interpulse interval *vs.* time) together by assuming that the beginning of the terminal phase in each case represented somewhat the same situation to the bat (perhaps a given range from the fly or perhaps a clear bearing) and that the bat's flight speed was uniform and constant, then mean pulse duration or interpulse interval at any point in time from the fly can be calculated. The first discernible decrease in mean pulse duration occurs at about 225 msec. before the beginning of the terminal phase (about 300 msec. before the end of the pursuit) (Fig. 3). Using mean interpulse interval, the first discernible drop may be at about 240 msec. and is certain by 225 msec. before the beginning of the terminal phase (Fig. 4). By these various criteria, therefore, a common value for the approach phase would be about 240 msec., for the terminal phase about 55 msec. Note that the entire terminal phase transpires in an interval comparable to a single interpulse interval of the search phase!

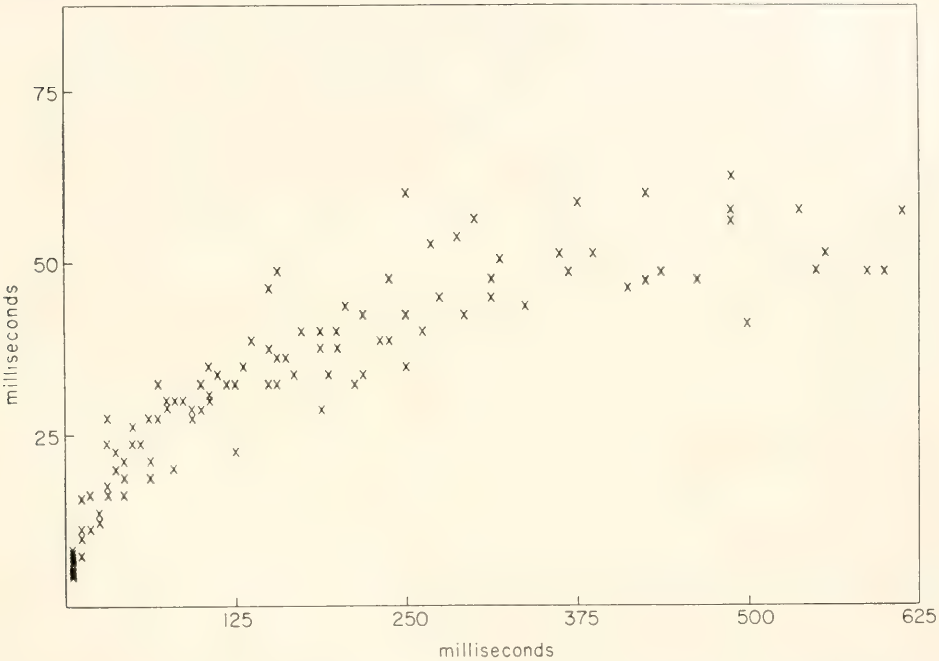

FIGURE 4. Nine fruit fly pursuits by *C. psilotis* have been synchronized and plotted together by assuming equality of situation at the beginning of the terminal phase. The duration of interpulse intervals (represented as events occurring at the beginning of the pulse preceding the interval) are plotted against time during the pursuit, setting the beginning of the terminal phase as zero. Note the transition immediately to the left of 250 msec. One msec. = 1.73 mm.

A complete analysis, knowing only the time separating the moving bat from its moving target, is impossible. Unfortunately, we did not have facilities for photographing these pursuits nor do we have an objective measure of the bat's flight speed. Time, however, can be converted to distance if several assumptions and approximations are allowed. First, an empirically chosen flight speed of 1.73 m./sec. has been assigned to this bat. Second, we assume the bat's velocity relative to the fly to be constant throughout the pursuit. And, finally, we assume that the pursuit ends with a capture or that the last pulse represents a zero point from which the distance separating the bat and fly can be calculated back for each previous pulse. We know the bat's velocity is not constant. During pursuits, bats appear to change direction as well as speed. We have no useful information about the flies but their directions of flight are probably random relative to the

TABLE I

Various parameters of insect pursuits by three closely related species of bats. The data for Pteronotus are from Novick (1963b); those for C. parnellii are from Novick and Vaisnys (1964).

	C. psilotis	Pteronotus	C. parnellii
Search phase			
pulse duration (msec.)	2.9–4.8	3.9–5.0	14–26
interpulse interval (msec.)	44–84	70–200	20–60
repetition rate (pulses/sec.)	17–18	10–12	*ca.* 14
Duration of pursuit			
time (msec.)	210–360	550–620	560–740
distance, calculated (mm.)	360–620	690–790	ca. 3,300
Approach phase			
duration in time (msec.)	150–290	350 (mean)	380–560
duration in distance, calc. (mm.)	260–500	440 (mean)	2,400 (mean)
number of pulses	5–9	3–10	7–10
pulse duration (msec.)	decreasing to 1.5–2.1	decreasing to 2.4–3.2	rising from 20-21 to 28–37 and then falling to 23–27
interpulse interval (msec.)	decreasing from 40–7	decreasing from about 50 to about about 25	fluctuating about 20–50 and then decreasing to 4–5
final repetition rate (pulse/sec.)	about 100	about 35	about 20 or slightly more
pulse-echo overlap (msec.)	*ca* 1.2	*ca.* 1.5	*ca.* 20
Terminal phase			
duration in time (msec.)	50–94	150–214	180–190
duration in distance, calc. (mm.)	90–160	190–270	800–900
number of pulses	11–18	27–39	9–12
pulse duration (msec.)	decreasing to 0.6–1.0	decreasing to 1.0–1.25	decreasing to 6–8
interpulse interval (msec.)	4.5–5	1.5	2.5
final repetition rate (pulses/sec.)	*ca.* 170	*ca.* 200 or more	*ca.* 80–100
pulse-echo overlap (msec.)	0.8–0.9	*ca.* 1.0	decreasing from 18 to *ca.* 5
Theoretical range (based on calculated pulse-echo overlap and flight speed)	400–700 mm.	680–860 mm.	3,800 mm.
Flight speed (mm./msec.)	1.73	1.25	1.5

bat (unless they take evasive action as do some moths; Roeder and Treat, 1961) and their speed is probably small compared with that of the bat. In any event, we have ignored the movement of the flies in these analyses. The flight speed of 1.73 mm./msec. was initially arrived at by plotting pulse duration *vs.* time from the end of the pursuit and arbitrarily seeking a flight speed which would yield a regular pattern of calculated pulse-echo overlap when time was converted to distance. The flight speed is consistent with known bat flight speeds and is independently confirmed by correlation (cited below) between first calculated pulse-echo overlap and first behavioral response as well as by its yielding regular patterns of pulse-echo overlap in all 9 pursuits. We have discussed, elsewhere, these extrapolations which have previously proved useful (Novick, 1963b; Novick and Vaisnys, 1964).

Fixing the bat's position at zero, then, at the end of the terminal phase, we have calculated its position at the beginning of each pulse during each pursuit. We find that the approach phases begin at about 500 mm. (360 to 620 mm.) from the fly and that the terminal phases begin from 90 to 160 mm. from the fly. The approach phase alone therefore occupies about 400 mm. (260 to 500 mm.) (Table I).

Having calculated the bat's position relative to the fruit fly, one can calculate the time taken for sound to cover the roundtrip distance (using 350 mm./msec. as the speed of sound). Knowing the pulse durations being used, one finds that the first pulse-echo overlap would occur at 230 to about 440 msec. or about 400 to 700 mm. before capture. The first pulse-echo overlap would normally be of very short duration (about 0.3 msec.). If the bat is flying at 1.73 mm./msec. and if the searching repetition rate is such that each pulse and its subsequent silent interval occupy about 60 msec., then the bat will have moved about 100 mm. between pulses. Neglecting the fly's movement, the round trip distance is decreased by 200 mm. Sound covers this distance in 0.6 msec. Thus, if the echo from a given fly just fails to overlap with one pulse, it will, on the average, overlap by 0.6 msec. with the next. Provided the bat did not respond to non-overlapping echoes by lengthening pulse duration (no apparent evidence in *C. psilotis*) the average first overlap would therefore be about 0.3 msec. The average second overlap would be about 0.9 msec., provided the bat had not already altered its behavior in response to the first overlap. If about 0.5 to 1 msec. of overlap is needed for the bat to perceive or take an interest in an echo, the first pulses providing such information occur at 340 to 670 mm. Thus, the first substantial pulse-echo overlap occurs at about the same time that the last normal interpulse interval is being produced (360 to 620 mm.). The coincidence of these values—the interpulse interval which is completely objective and the calculated first significant pulse-echo overlap—tends to justify the assumptions made above which allowed these extrapolations. Similar relationships have been reported in *Pteronotus* and *C. parnellii* (Novick, 1963b; Novick and Vaisnys, 1964).

Specifically, the first calculated pulse-echo overlap precedes the first behavioral response by 34 to 150 msec. This puts vague limits on apparent response time. The range of values may well reflect the extrapolation error. In *C. parnellii,* where flight speed was objectively recorded, a possible response time of 130 to 150 msec. was reported (Novick and Vaisnys, 1964). Grinnell's (1963a, 1963b) studies of brain responses to acoustic stimuli in anesthetized vespertilionid bats suggest that cortical decisions may be involved in these initial echo recognitions. In subsequent portions of pursuits, time seems to be lacking for higher level judgment.

The first, second, or third pulse following the first calculated pulse-echo overlap is distinguishably shorter than the searching pulses. Thereafter, pulse duration decreases close to linearly when plotted *vs.* time or distance separating the bat and fly. The slope of this line varies in different pursuits from 1 msec. of shortening of pulse duration per 100 mm. of apparent reduced separation to 1 msec./350 mm. These different slopes may indicate different closing speeds of bat on insect (as, for example, if the insect is flying directly toward rather than directly away from the bat). In all pursuits, however, the relationship is linear during the approach phase. Pulse duration drops from average values of about 4.0 to 4.2 msec., initially, to values of 1.5 to 2.1 msec. at the beginning of the terminal phase.

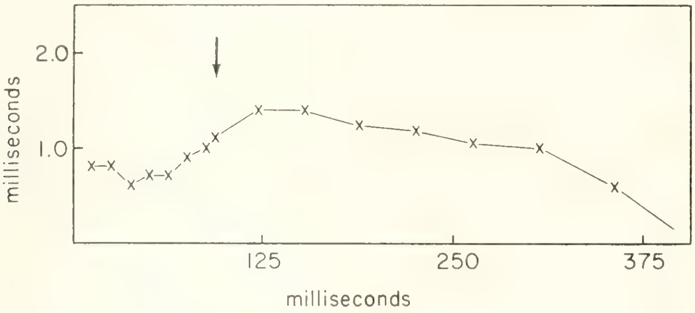

FIGURE 5. Pulse-echo overlap in the longest, recorded single fruit fly pursuit by *C. psilotis*. Note the rapid rise of pulse-echo overlap to values of 1.0 to 1.4 msec. during the approach phase and the shift to values of 0.6 to 0.8 during the terminal phase. The arrow indicates the beginning of the terminal phase. The calculated pulse-echo overlap is shown for each pulse during the approach phase but a representative value is shown for each 12.5 msec. during the terminal phase to increase clarity.

As has been the case in *C. parnellii*, pairing of pulses in the late approach phase sometimes occurs. That is, two pulses of close to the same duration are followed by two of shorter but almost equal duration and so on. Such pairs may be the functional units. The governing feature, here, may be reaction or decision time (that is, that decisions concerning revision of pulse duration take longer to make than the time available between single pulses—about 25 msec. or less), or pairs may actually be functionally desirable (as, for example, to assess direction by exploiting the movement of the head between the two pulses which, because of beaming and directionally tuned ears, would alter the echo intensity).

The speed of sound is constant, the bat's flight speed has been assumed constant, and pulse duration *vs.* time or distance drops linearly. When one calculates the successive pulse-echo overlaps, therefore, these prove to remain constant throughout the approach phase after an initial rapid rise. Thus, at 390 mm. from the fly, where all nine pursuits have calculated overlap values, the range of overlap is 0.4 to 1.7 msec. with a mean of 1.0 msec. At 300 mm., the range is 0.6 to 1.5, mean 1.1 msec.; at 170 mm., range 0.9 to 1.6 msec., mean 1.2 msec. In individual pursuits, the value is remarkably constant. Variations of less than 0.5 to 0.6 msec. are the rule during the approach phase. The characteristic individual approach phase overlap levels of the respective pursuits are 1.1, 0.9, 1.2, 1.6, 1.2, 0.9, 1.1, 1.2, and 1.2 msec. (Fig. 5).

During the approach phase, then, pulse duration drops off linearly from about 4.0 to 4.2 msec. to about 1.4 to 2.1 msec. as the pulse-echo overlap levels out at about 1.2 msec. During this time, interpulse interval also drops sharply but not linearly. Search phase interpulse intervals of about 56 msec. yield to intervals averaging about 40 msec. between 200 and 270 msec. before the beginning of the terminal phase; to 31 msec. between 95 and 125 msec.; to 21 msec. betwen 30 and 60 msec.; and to about 12 msec. between 13 and 30 msec., respectively, before the beginning of the terminal phase. The last interpulse interval of the approach phase or, perhaps, the first of the terminal phase is about 7 msec. in duration with little variation from one pursuit to another (Fig. 4).

The repetition rate, about 17–18 pulses/sec. during the search phase, has increased to about 110/sec. by the end of the approach phase. The approach phase in *C. psilotis* varies in these analyzed pursuits from 5 to 9 pulses in length.

The terminal phase in *C. psilotis* is grossly distinguishable from the approach phase. The interpulse interval drops immediately to a minimal value of about 4.5 to 5 msec. and remains at that level throughout. The pulse duration also drops from initial values of about 1.3 to 1.9 msec. to terminal values of 0.6 to 1 msec., without a dramatic shift from the previous linear drop of the approach phase until values of 1.0 to 1.3 msec. are achieved, after which the pulse duration curve flattens out and appears to approach a limit. Excepting one example, there were from 11 to 18 pulses in the terminal phase. In the exceptional case only 7 pulses appeared. This might have represented a rapid catch or a miss. The terminal pulse repetition rate, on the average, is about 170 pulses/sec.

The transition from approach phase to terminal phase is generally nonambiguous and is most clearly defined by a break in the interpulse interval *vs.* time curve. The interval just before the transition one is from 2 to 4 times as long as the interval just after the transition one. During the terminal phase, calculated pulse-echo overlaps are shorter than during the approach phase but again are uniform and regular with values ranging from about 0.6 to 1.3 msec. At a distance of 90 mm., the overlap averages 0.9 msec.; at 65 mm., it averages 0.8 msec.; at 45 mm., 0.8 msec.; and at 20 mm., 0.9 msec. (Fig. 5).

Sound spectrographs of pulses selected from search, approach, and terminal phases universally show a fundamental initially of about 21 kcps accompanied by its second, third, and fourth harmonics of 42, 63, and 84 kcps, respectively. The fundamental frequently appears faint. Either the second or, more often, the third harmonic is at the highest amplitude, especially during the terminal phase (Fig. 1).

The initial frequency varies slightly from pulse to pulse (perhaps by 1 kcps), but does not vary systematically during a pursuit. The last pulses are essentially like the first in frequency components.

During the course of a pursuit, however, the frequency pattern changes. In every case, the frequency drops during a pulse. The fundamental in search phase pulses drops to 17 kcps at the end of the pulse while the harmonics drop to multiples of 17. The same 21 to 17 kpcs fundamental frequency drop seems to characterize all of the pursuit pulses. The slope of the drop, however, changes. In the search phase, the initial 1.3 to 1.5 msec. of each pulse seem to be close to constant in frequency. The final 1.0 to 1.3 msec. or so seem also to be constant in frequency or, at least, of very shallow slope. The frequency drop occurs during the central part of the pulse (Fig. 1).

The first apparent change in the frequency pattern is a change in slope of the final portion of the pulse which shifts, from being constant in frequency, to fusing more and more with the central pulse portion, but can still be distinguished by an inflection point in the early terminal phase, after which it can no longer be clearly identified.

The initial constant frequency portion shortens during the approach phase and also starts to have a frequency drop, though at a different slope from the rest of the pulse. The drop becomes more marked and fuses with the rest of the pulse early in the terminal phase. The central, frequency modulated portion remains much the same throughout a pursuit.

Thus, during the search phase, the frequency pattern has a lazy Z shape (Fig. 1). During the approach phase, the curve becomes sigmoid, and during the terminal phase looks like a slash mark. The frequency *vs.* time curve even in the terminal phase may not be a straight line, but the resolution of our equipment does not reveal any clear and regular inflection points.

Discussion

The constant or almost constant frequency portions of the search and early approach phase pulses may provide markers for distance identification. In any event, if our calculations are approximately correct, the usual pulse-echo overlap, here about 1.2 msec., would tend to consist of an echo of constant fundamental frequency of about 21 kpcs overlapping with an outgoing constant frequency portion of 17 kpcs. During the search phase, at least three kinds of echoes could easily be distinguished. Those just barely overlapping (about 1 msec.) would give a simple situation of two separate but constant frequency components. Closer objects would give pulse-echo overlaps involving the frequency modulated parts of the pulse and echo as well as the constant frequency parts. More distant objects would give no pulse-echo overlap. Thus, "too much" and "too little" overlap could be identified easily.

In the approach phase, the constant frequency portions are systematically changed. Soon they, too, show a dropping frequency and by the early part of the terminal phase are no longer identifiable. It should be possible for such differing frequency slopes to provide intra-pulse markers which could be used for distance measurement or for the assessment of closing speed relative to the pursued target. If these portions are, indeed, regular, then the bat may well use the pulse-echo overlap as a way of deciding whether it is at the expected distance from its target, or closing in on it faster or slower than anticipated, simply by noticing whether the overlap occurs during one portion of the pulse or another.

During the terminal phase, the typical pulse-echo overlap shifts to 0.8 msec. There is no clear marker at this time, though a poorly resolved change in frequency may be present. If not, what does the constant pulse-echo overlap here signify and how is it measured and regulated? No answer is presently available.

Thus, the possibility presents itself that one purpose served by pulse-echo overlap is to measure distance and, perhaps, changing distance. Such a measurement would be independent of any knowledge of the speed of sound or the ability to discriminate short intervals of time. The initial detection in the search phase would simply give the information that there was a target at the standard specific detection range. Later information would confirm satisfactory closing speed.

If, indeed, the search pulses are designed with initial and final constant frequency portions in order to facilitate the recognition of echoes from a given useful hunting range, we still have to account for the frequency modulated character of the central portion. If the beginning of the pulse is to be different from the end, a frequency change must occur somewhere. The frequency change here may represent only that. Its slope may, instead, be the natural slope resulting from the relaxation of the laryngeal muscles in this bat. Other ways in which this frequency change may be exploited remain obscure.

The previous records of *C. parnellii* have been reexamined in a search for coincidence between pulse-echo overlap and frequency inflection points. In *C. parnellii,* almost the entire pulse is of constant frequency. Only the final 1 to 2 msec. show a frequency drop. Such a drop could well allow for initial detection during searching but would soon be only a small part of the typical 20-msec. overlap. In *C. parnellii,* note, however, that the typical pulse-echo overlap of 20 msec. equals the usual search phase pulse duration of 20 msec. and the 1-2 msec. frequency drop equals the frequency drop seen in *C. psilotis.* These may be coincidental but seem of possible interest. In any event, there is only an incomplete parallel with *C. psilotis.* Reexamination of the old records of *Pteronotus* does not permit more than a statement that the frequency pattern changes during pursuits. New examples will have to be recorded and analyzed.

In summary, all pulses seem to include about the same amount of frequency drop (perhaps, a result of the mode of sound production; Novick and Griffin, 1961). Search phase pulses are each, during an initial and a final 1.5-msec. portion, constant or almost constant in frequency. These constant frequency portions shorten during the pursuit and also incorporate, more and more, the standard frequency drop so that any clear separation between the initial and final pulse portions *vs.* the central pulse portion disappears by the early part of the terminal phase. These frequency patterns and their changes could provide target distance information during the pursuit by changing pulse-echo contrast. Such frequency changes would allow a three-quantity judgment of distance—just right, closer than expected, or further than expected. Such judgments could be translated into prediction of the target's relative flight path.

The data so far accumulated on the parameters of echolocation during insect pursuits by chilonycterine bats are summarized in Table I. Some of the data from previous papers have been slightly recalculated or restated.

The closeness of these species of bats is exemplified by the decision of Burt and Stirton (1961) to include *Chilonycteris* in the genus *Pteronotus.* These authors felt that morphological considerations did not justify generic status, but other authors have disagreed and retain the two genera as we have chosen to do here. All of these closely related bats were recorded pursuing a mixed group of *Drosophila* while flying in the same laboratory room. Thus, the targets and the auxiliary orientation problems, as well as the bat species, were similar. Flight speed, sound output design (frequency, frequency pattern, pulse duration, intensity, and interpulse interval) differed and presumably other parameters, not yet assessed, also varied. A comparison of the three performances, at this point, however, raises interesting but not yet answerable questions.

While searching, *C. psilotis* produces slightly shorter pulses than *Pteronotus*. Those of *C. parnellii* are 5 times as long. If pulse-echo overlap is needed for detection, pulse duration sets maximum range. Presumably flight speed also affects maximum practical range as must, indeed, pulse intensity. We can only observe for the moment that *C. parnellii* flies fastest and produces the loudest and longest pulses. Seemingly as a result, *C. parnellii* shows a fruit fly detection range of over 3 meters. *C. psilotis* produces the shortest pulses of these three species, giving it the smallest range of detection. This shortest pursuit distance, combined with faster flight than *Pteronotus*, results in the shortest pursuit duration (about ½ that of *Pteronotus* in time as well as in the number of pulses). Such a short pursuit involves many fewer decisions about the position of the fly and the bat's own flight control. Fewer decisions, of course, would seem to have been adequate since all of these bats seemed successful in their pursuits. If the range is small, the fly moves over a shorter distance during the pursuit. If the range is smaller, perhaps there is greater certainty about the position of the fly—its direction principally. The greater the range, the more problems would arise from confusing or ambiguous echoes. But probably, the greatest reason for the vastly greater number of pulses during *Pteronotus*' pursuits than during those of *C. psilotis* is that the bat flew more slowly and simply had to follow the fly for a longer time. One might reasonably ask why all these bats would not have hit on the same formula of pulse duration, intensity, and range. Flight speed itself might be the important difference but the size and flight habits of the common prey species might be even more important. All of these bats commonly occupy the same caves in large numbers. They seem to have very similar climatic demands. One might guess that they would hunt different insect populations and thus avoid competition. Insufficient information on their feeding habits precludes further speculation on this now. The striking anatomical difference in the wings of *Pteronotus* (which originate from the midline of the back as opposed to lateral attachment in almost all other bats) implies different flight habits.

The differences in interpulse interval (and, hence, repetition rate) may well reflect flight speed. In all three species, the repetition rate is such as to provide an apparently significant pulse-echo overlap from objects just beyond range of overlap with the previous pulse. Again flight speed seems to be a prime variable but there is also an apparent difference in the amount of overlap which draws the bat's attention. *C. psilotis* moves about 100 mm. between pulses, *Pteronotus* about 120 mm. and *C. parnellii* about 300 mm. Thus, objects just beyond overlap range from one pulse will yield about 0.6, 0.7, and 1.7 msec. of overlap, respectively, with the next pulse. The behavioral responses of each of these species suggest that this amount of overlap is enough to attract attention. The spacing of search phase pulses produced by each of these species is, however, surprisingly similar, aside from the above considerations. Since the speed of sound is so great compared even with the swiftest of these three species, the common pulse spacing of about 50 to 75 msec. may simply reflect the length of the central nervous system path involved in searching decisions. Grinnell's (1963a, 1963b) recordings from vespertilionid bat brains may cast some light on these time relations.

The time consumed in an insect pursuit is remarkably similar in *Pteronotus* and *C. parnellii* but only ½ as long in *C. psilotis*. In *C. psilotis* both the approach and terminal phases are shorter than in the other two bats. The approach phases in *Pteronotus* and *C. parnellii* are roughly equal in length as are the terminal phases. The savings in the approach phase for *C. psilotis* seem to come from the more immediate and consistent shortening of the interpulse intervals. The number of pulses is not noticeably different from that of the other bats. In the terminal phase, *C. psilotis* uses far fewer pulses than *Pteronotus* (11–18 *vs.* 27–39) and, of course, much shorter pulses than *C. parnellii*. Possibly the promptness of change in interpulse interval during the approach phase in *C. psilotis* is a direct result of the short range at which it detects fruit flies and the resultingly more clearly defined target.

The distance covered in the approach phase is similar in *C. psilotis* and *Pteronotus* since *C. psilotis* flies somewhat faster. A bat of such size may require this minimal distance in order to align itself properly to intercept a fruit fly. But in the terminal phase, the distance is markedly shorter in *C. psilotis* (90–160 mm. *vs.* 190–270 mm.). This may reflect more accurate identification of the insect's position by the end of the approach phase in the case of *C. psilotis* or may imply more information per pulse or may simply result from the greater flight speed or shorter range. That is, the difficult problem may be to line up on the fly during the approach phase. The task of the terminal phase may simply be to keep the fly's position identified while one covers the intervening distance. It is possible that the differences which we see in length of pursuit may simply reflect the relative adaptedness of *C. psilotis* to detecting, pursuing, and/or catching fruit flies.

In both *C. psilotis* and *Pteronotus*, pulse duration decreases sharply during the approach phase. In both, the decrease is close to linear *vs.* time, implying that the bat is measuring distance from the target. If pulse duration were adjusted so as to give essentially equal amounts of pulse-echo overlap, then distance would, in effect, be measured. We have no direct evidence so far concerning how well a bat can discriminate time intervals or lengths of overlap. See discussion above.

There has previously been very little direct evidence of proportional measurement of distance in bats. Search phase pulse durations are presumably set so as to give pulse-echo overlap at a desirable hunting range. Unfortunately, our analyses are limited by having studied only one kind and size of target in the same room. In *C. parnellii*, the pulse duration during the approach phase rises and then falls, but in doing so accomplishes the same result as that in the other two bats—constant pulse-echo overlap. Here, too, a measurement of distance is implied. And in the terminal phase, pulse duration falls linearly *vs.* time.

Apparently either fixed amounts of pulse-echo overlap are useful during the approach phase or some quantity proportional to pulse-echo overlap is being held constant purposefully. The degree of overlap is comparable in *C. psilotis* and *Pteronotus* but is some 15 times longer in *C. parnellii.* This is a striking difference in such closely related bats but its significance eludes us.

During the approach phase, the longest interpulse intervals in all of these bats are about 40–50 msec., followed by or alternating commonly with intervals of about 25 msec. before shortening sharply to values of about 5–7 msec. at the beginning of the terminal phase. Such regularity suggests that these interpulse intervals represent the reaction times for decisions of decreasing complexity as well as the need for increasingly frequent information. These two features—more frequent information *vs.* more thorough digestion and judgment of the information may well be segregated during the pursuit. In a pursuit which in all consumes substantially less than a second, *Pteronotus* may emit over 40 pulses. A tempting suggestion would be that the bat, detecting an interesting echo for the first time, might turn on an ordered and predetermined sequence of pulses of varying parameters and run through such a sequence without having to make more than a few intermediate decisions. Examining the various pursuits, however, we find that they are similar but non-congruent in almost every respect. The different number of pulses (total and in each phase), the different pulse durations, repetition rates, and total pursuit durations all imply a large number of intermediate decisions. At least two decisions, the duration of a later pulse and of a later interpulse interval, seem necessarily to be made after at least each of the early approach pulses. Later, the frequent pairing of pulses in terms of duration and the final steadiness of the interpulse intervals in the terminal phase may indicate a reduction in the number of decisions being made per pulse. The number of intermediate decisions involved in arriving at the desirable pulse duration and interpulse interval cannot now be assessed. But, in any event, about 50 to 60 msec. seem to be available in the early approach phase, about 10 msec. in the late approach phase, and about 5 msec. in the terminal phase in *C. psilotis* and *Pteronotus.* The times are slightly different in *C. parnellii.* Information is rapidly accumulating on the times involved in acoustic pathways in the central nervous system of bats but does not seem ready to permit useful speculations on the length of the paths involved here (Grinnell, 1963a, 1963b).

Interpulse intervals are essentially the same during the terminal phase in both *C. psilotis* and *Pteronotus* and may well be at the limit of muscle and nerve physiology in these as well as in *C. parnellii* (2.5 msec.). A final repetition rate approaching or exceeding 200 pulses/sec., as in *C. psilotis* and *Pteronotus,* clearly implies rapid contraction and relaxation of sound-producing musculature (Novick and Griffin, 1961; Revel, 1962). Repetition rates of this order of magnitude seem to be the limit in bats (Griffin, 1958). In addition to the physiological limitations of the musculature and nerves involved, the limit may also be imposed to prevent overlap of echoes with subsequent pulses.

Henson's (1964) recent studies of cochlear microphonics and stapedius muscle activity in *Tadarida* (Molossidae), a free-tailed bat, have suggested additional possible interpretations of some aspects of repetition rate in insect pursuits. In this bat, the stapedius muscle may contract some 6 to 15 msec. before pulse emission, when the pulse repetition rate is 50/sec. or less. At higher repetition rates, 60 to 80/sec., stapedius muscle contractions occur 4 to 8 msec. before pulse emission. At even higher repetition rates, 100–140/sec., stapedius contraction is continual. Such contractions reduce cochlear microphonics to a degree that implies about 20 db attenuation of incoming sound. Stapedius relaxation appears to be initiated within 1–2 msec. and complete by 10 msec. after the beginning of pulse emission at low repetition rates. At the higher repetition rates, the stapedius may remain contracted for up to 40 msec. after the end of the pulse train.

At low repetition rates, stapedius relaxation occurs rapidly enough for echoes from nearby objects to evoke larger cochlear microphonic potentials than do the outgoing pulses (Henson, 1964). Now, during the search phase, when echoes would presumably be faint and would occur unpredictably, there might well be advantage to having full stapedius contraction during the bulk of the outgoing pulse, with considerable relaxation at the time of echo reception (for example, during the last millisecond in *C. psilotis*). This design feature would interact with pulse duration since the pulse would have to last long enough to allow some stapedius relaxation. During the approach phase, the intensity and timing of the echo can be anticipated to some degree but may well have to be assessed carefully and, of course, the echo will still be relatively faint since the fly is from 100 to 500 mm. away. Thus the repetition rate may be kept low to allow maximum contraction of the stapedius during pulse output and relaxation during echo input, yielding protection and relative amplification alternately. By the end of the approach phase, at least two general features will have changed. The echo will have been substantially identified—the target located—and the echo will be predictable in time. Such anticipation of echo timing can improve perception of the echo. In addition, the target will be close, less than 150 mm., so that the signal will be physically more intense. These advantages may well permit sacrifice of stapedius relaxation and substitution of high repetition rate. At such close range auxiliary factors reducing the apparent intensity of the outgoing sounds (acoustic isolation of the ear capsule, directional pinnae, etc.), central facilitation (Grinnell, 1963a, 1963b), and increasing echo intensity might all combine to give satisfactory signal:noise ratios. Note, too, that the intensity of pulses during the search and approach phases ought to be greater than during the terminal phase because of the greater range. There is little objective evidence of absolute intensities during these pursuits since the distance and orientation of the bat relative to the microphone have not been recorded. Bats which routinely must hunt at short target ranges, however (Nycteridae and others), use low intensity pulses (Novick, 1958) while bats which routinely hunt at long distances (*Rhinolophus* and *C. parnellii*) use high intensity (Novick and Vaisnys, 1964). High intensity output would also favor maximum stapedius contraction and relaxation during the search and approach phases while less contrast would be required during the terminal phase.

Lastly, one should consider whether the frequency pattern exemplified by *C. psilotis* reflects simply coordination of the cricothyroid muscle with the stapedius muscle. Novick and Griffin (1961) have shown that cricothyroid muscle action potentials can be recorded, from several genera of bats, which have timings relative to the emitted pulses very similar to those of *Tadarida* stapedius muscles (Henson, 1964). If these two systems are designed to work together, the rapid responses required may have led to development of reflex association of the muscles involved so that the frequency pattern and/or the duration of the pulses during the various portions of the pursuits may be set by the needs of stapedius contraction and relaxation.

In summary, repetition rate during search and approach phases might be set to allow time for stapedius contraction and relaxation, in order to clarify echo perception. In the terminal phase, when echoes would be more intense, stapedius relaxation appears to be surrendered in favor of rapid repetition rate.

What determines the ultimate pulse duration in the terminal phase in these three species has not been clarified. In *C. psilotis* and *Pteronotus,* pulses of 1 msec. and shorter characterize this phase. Shorter pulses occur in insect pursuits in vespertilionids (Griffin, 1962; Webster, 1963) and in the general emissions of many other bats (Novick, 1958, 1963a), but the pulses seen here are, nevertheless, very short and may represent the best that the physical apparatus of these bats can deliver. On the other hand, in these three species, we have substantial circumstantial evidence that pulse-echo overlaps are essential to insect pursuit and that the useful amount of pulse-echo overlap in *C. psilotis* and *Pteronotus* is about 1 msec. If, indeed, they depend on such overlap, the limits on pulse duration are set. The vespertilionids apparently avoid overlap by using pulses as short as 0.3 msec. or less. All insectivorous bats, vespertilionids as well as chilonycterines, must be very close to an insect ultimately in order to capture it. We do not yet have sufficient information on the actual range between the ear and the mouth and the insect in any species of bat at the moment of capture though there are well documented examples of bats capturing insects in their wing membranes or interfemoral membranes (Webster and Griffin, 1962). Such cases involved large insects and usually unnatural situations, however, and did not include our chilonycterines. But if a vespertilionid can avoid pulse-echo overlap up to the last moment by using pulses of 0.3 msec. and a chilonycterine uses pulses of about 1 msec., we may conclude, independently of other calculations, that in the last moments of a pursuit, *C. psilotis* and *Pteronotus* are still getting about 0.7 msec. of pulse-echo overlap.

Even though the terminal phase occupies only a very short time, during which the bats travel only a short distance compared with the distance covered in the approach phase, pulse duration must still decrease (and, indeed, it does) if pulse-echo overlap is to be held steady. At the very end of the terminal phase, pulse duration may flatten out or even fluctuate a bit. This may be evidence of the relatively great effect of the movement of the fly at close range and/or variation in the physical site of capture (wing, mouth, etc.). In *C. parnellii,* where there would seem to be greater leeway to shorten pulse duration without losing pulse-echo overlap or overtaxing the sound producing mechanism, the pulse duration seems to continue to decrease linearly (in pairs) throughout the terminal phase.

In all, these comparative data cast some light on the design of pulse duration, pulse repetition rate, pulse number, detection range and pursuit duration in these three species of bats during searching and during insect pursuits. We need more information on frequency pattern, intensity, flight speed, and the echolocation of targets of different sizes and velocities in order to reach more definitive conclusions. We would also benefit from more information about the natural prey and hunting habits of each of these bats.

SUMMARY

1. Elements of the acoustic orientation of *Chilonycteris philotis* during insect pursuits have been observed and analyzed. Such pursuits can be subdivided into search, approach, and terminal phases. The search phase is characterized by pulses of about 4 msec. duration repeated at a rate of about 18/sec. On detection of an insect, apparently by pulse-echo overlap (at a typical range of about 400–700 mm.), the approach phase begins, characterized by shortening (linear *vs.* time) pulse duration (to about 1.5 to 2.1 msec.) and shortening interpulse intervals. The approach phase (which lasts about 150–290 msec.; 5–9 pulses) ends in a transition to the terminal phase—a rapid sequence of short (1-msec.) pulses produced at a rate of about 170/sec. The terminal phase lasts about 50–94 msec. and includes 11–18 pulses. Constant pulse-echo overlap of about 1.2 msec. characterizes the approach phase, implying distance measurement and overlap utility. During the terminal phase, pulse-echo overlap appears to be set at about 0.8 msec. The pulses of *C. psilotis* consist of a fundamental frequency, initially of about 21 kcps, accompanied by its second, third, and fourth harmonics. During the search phase, the initial and final 1.5 msec. of each pulse are of constant frequency but the central portion shows a frequency drop (for the fundamental about 4 kcps). During a pursuit, the constant frequency portions are shortened and then fused into the frequency modulated portion so that they are no longer recognizable beyond the early part of the terminal phase. Such a frequency pattern suggests possible mechanisms for recognizing and using pulse-echo overlaps.

2. The parallel parameters of *Pteronotus* and *Chilonycteris parnellii* pursuits, previously studied chilonycterine bats, are tabulated and compared with *C. psilotis*.

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REPRODUCTIVE CYCLE OF MYA ARENARIA IN NEW ENGLAND

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Knowledge of the reproductive cycle in the soft-shell clam, *Mya arenaria* L., is essential to an understanding of larval production and ultimately of the abundance of this commercially important bivalve. By following the progress of gonad and gamete development in *Mya* throughout the year, the times and duration of spawning can be determined—information useful in the management of this fishery.

Although the literature contains numerous references to the times of spawning, information about gonadal changes in *Mya* is fragmentary or incomplete. We found the smear technique, used by Battle (1932), unreliable for determining gamete development because important details seen in the histological preparations were obscure in fresh, unstained smears. Coe and Turner (1938) described the development of juvenile *Mya* and gametogenesis from histological preparations. Unfortunately, their study indicated only the beginning of spawning and gave no data on its duration. In a preliminary report, Rogers (1959) described gonad development in Chesapeake Bay, Maryland, during the summer and fall. He observed a fall spawning period and ventured an opinion that a spring spawning also occurred, but he had not completed a full year of observations. Pfitzenmeyer (1962) also reported preliminary histological observations to support a conclusion that Maryland clams have two separate maturations.

Plankton studies by various investigators have revealed both annual and biannual periods of larval abundance. Both Stevenson (1907) and Stafford (1912) reported their observations of larvae as most numerous during a single period of time. Sullivan (1948) used the term "broods," possibly to describe successive large groups of larvae, but did not mention intervals between the groups when none were caught. Landers (1954) observed both an early-major and later-secondary larval occurrence. Between the two swarms, larvae disappeared for intervals of one to two months. Pfitzenmeyer (1962) observed an early-secondary and later-major larval occurrence. Larvae were not caught for intervals of three to four months between these biannual swarms.

Inferences about the frequency of spawning have also been made from observations of the first appearance of newly settled juveniles in the flats. Successive ripening and spawning periods during a single reproductive cycle were hypothesized by Belding (1930) to explain the "not uncommon" occurrence during any one year of "two distinct sets" in areas on the southern shores of Cape Cod. Pfitzenmeyer (1962) used special collectors in Chesapeake Bay and obtained two distinct sets of juvenile clams each year. A period of two to three months when none were caught separated the biannual settings.

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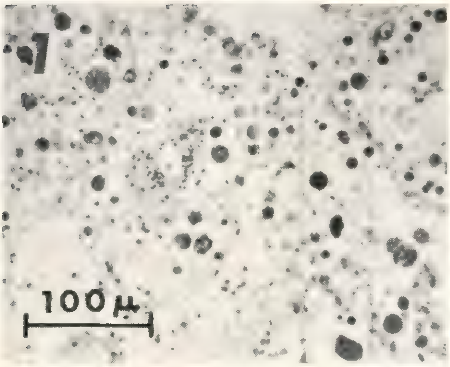
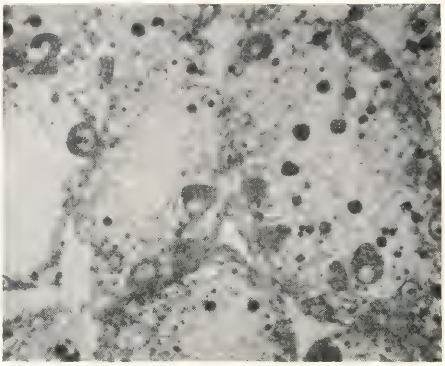
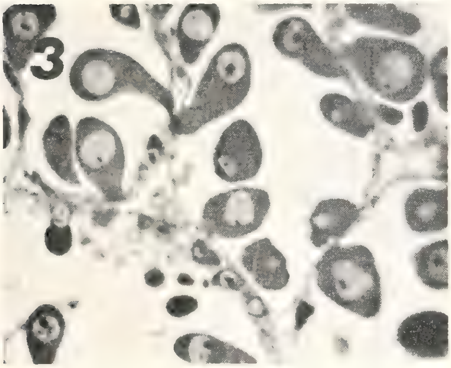
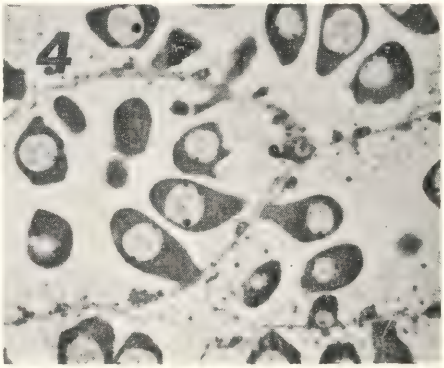
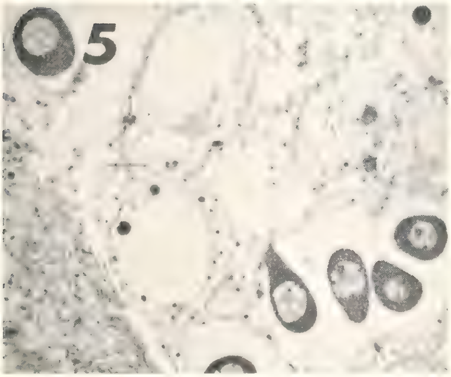










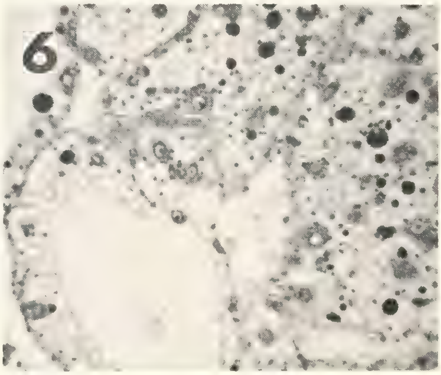


FIGURE 1. Section of gonad tissue from female clam, inactive phase of reproductive cycle. Note the small, dark phagocytes in the center of the alveolus, nutritive inclusions scattered within the follicle cells, and a large, round, almost completely cytolyzed ovocyte. To permit a comparison of cell size during maturation, all the photomicrographs were taken at the same magnification and a scale (100 μ) is provided.

FIGURE 2. Same, from female clam in active phase, showing early stages of ovogenesis.

FIGURE 3. Same, from female clam in active phase, showing later stages of ovogenesis.

Unfortunately, no certain conclusions about the nature of the reproductive cycle itself can be drawn from observations of either planktonic larvae, or newly metamorphosed set, since several seasonal peaks of abundance may appear from spawnings resulting from a single annual reproductive cycle. While observations of larval swarms can provide corroborative evidence, the annual frequency and duration of the reproductive cycle of clams can best be learned from periodic histological examinations of their gonad tissue. This paper presents the results of such studies made on clams from several New England areas.

METHODS

Collections of clams were obtained periodically from the following New England areas north of Cape Cod. Plum Island Sound clam flats in Newbury, Massachusetts, were sampled twice each month from April, 1951, through February, 1953; flats near Boothbay Harbor, Maine, from February to November, 1961 (except April); and flats in Sorrento, Milbridge, Roque Bluffs, Cutler, Lubec, Pembroke, and Robbinston, Maine (collectively designated as eastern Maine in the text), each month from September, 1959, through December, 1960. The clams were dug from the middle of the intertidal zone in the eastern Maine and Boothbay Harbor areas, but throughout the whole intertidal zone in Plum Island Sound because the clams were not abundant. Miscellaneous collections were also obtained from Falmouth and Woods Hole, Massachusetts. These samples from areas on the south side of Cape Cod provided valuable supplementary data. Each sample included approximately 10 clams, $1\frac{1}{2}$ to 3 inches in shell length.

Within a few hours after field collections were made, the anterior third of the visceral mass of each clam was removed and preserved in Bouin's fixative. Standard techniques of dehydrating in alcohol, imbedding in paraffin, sectioning at 10 μ, and staining in Delafield's hematoxylin and eosin were used to prepare slides of the gonad tissues.

A microscopic examination was made to assign each specimen to a category which represented the gonad condition. The proportion of clams in each category, regardless of sex, was recorded for the individual samples. Although minor differences were observed, data on the clams from the seven eastern Maine sample areas, as well as the twice-a-month samples from Plum Island Sound, were combined each month to simplify presentation. Since the combined samples had no single collection date, a date midway between the first and last collection dates was chosen to represent each month's observation of gonad condition.

CATEGORIES OF GONAD CONDITION

Female gonads

Except for certain pre-meiotic stages, ovocyte maturation occurs not in the ovaries, but after the eggs are shed (Raven, 1958). Therefore, tissues could not be classified according to the meiotic stages of the ovocytes. Instead, arbitrary

FIGURE 4. Same, from female clam in ripe phase. Note the amphinucleoli in the centrally located ovocyte.

FIGURE 5. Section of gonad tissue from female clam in partially spawned phase.

FIGURE 6. Section of gonad tissue from spent female clam. Note the small ovocytes at the periphery of the alveoli.

categories of development were distinguished by dividing the more or less continuous reproductive process into five phases. These are described below.

1. *Inactive phase*

At certain times of the year the rate of gonad activity becomes so low that changes in appearance of the gonad tissues in successive samples are imperceptible. Therefore, this state of relative quiescence is hereafter termed the inactive phase of the reproductive cycle. The term, however, is used for convenience and is not meant to imply that no activity at all is taking place. Small ovocytes occur at the periphery of alveoli (Fig. 1). A round nucleus in the ovocyte contains a conspicuous basophilic nucleolus and is encircled by an irregularly shaped cytoplasm. Vacuolated follicle cells that completely imbed the ovocytes sometimes fill the lumina of alveoli. Inclusions, apparently nutritive in function (Coe and Turner, 1938), frequency occur in the follicle cells. Small migratory cells sometimes appear between alveoli and within the lumina of alveoli. These migratory cells are probably phagocytic for they surround cytolyzed, unspent ovocytes.

2. *Active phase*

That part of ovogenesis which takes place within the gonad tissue and where definite quantitative and qualitative changes in the ovocytes are perceptible is designated as the active phase of the reproductive cycle. Enlarging ovocytes grow between the follicle cells toward the centers of alveoli during the early stages (Fig. 2). These ovocytes, which may be sub-conical, hemispherical or cylindrical in shape, are characteristically rounded at their apices and have broad cytoplasmic bases attaching them to the wall of the alveolus. The nuclei measure from 8 to 16 μ and average 13.3 μ in diameter.

Subsequent growth produces large, round ovocytes with constricted cytoplasmic bases (Fig. 3). The free ends of ovocytes extend beyond the basal membrane of follicle cells into the lumina of alveoli. The nuclei measure from 15 to 23 μ and average 19.2 μ in diameter. These ovocytes occur late in the active phase.

The onset of ovogenesis is difficult to determine with precision, as pronounced changes in staining reactions do not occur. However, the slight enlargement of many ovocytes, the more regular shape of their cytoplasm and protrusion toward the alveoli centers are visible indications of growth activities. Thereafter, the growth of ovocytes and tissue changes are more definite.

3. *Ripe phase*

In ripe clams, a very slender stalk may connect many of the largest ovocytes to the basal membrane (Fig. 4). Other ripe ovocytes appear as round cells in the lumina of alveoli, as if free of attachment to the basal membrane. The nuclei of these largest ovocytes contain amphinucleoli (Allen, 1953), each consisting of an almost transparent nucleolus and a small opaque nucleolinus. The amphinucleolus resembles a signet ring in some sections because the smaller nucleolinus occurs at the periphery of the nucleolus. The nuclei of ripe ovocytes measure from 20 to 31 μ and average 28.2 μ in diameter. These large ovocytes fill the lumina of alveoli and are usually more numerous than less developed ovocytes.

4. *Partially spawned phase*

In partially spawned clams, gonad tissues contain a few ripe ovocytes in some of the alveoli (Fig. 5). Small ovocytes are imbedded in follicle cells at the periphery of empty alveoli. Nutritive inclusions appear in many of the follicle cells. An absence of ripe ovocytes in many alveoli and the cessation of ovogenesis in all alveoli is indicative of the partially spawned condition.

5. *Spent phase*

Follicle cells form a thin layer covering the basal membrane of alveoli in some spent tissues, while in others they fill the alveoli (Fig. 6). Spent individuals can be distinguished from those in which no gametogenesis has taken place at all by the presence of a few unspent ovocytes in the early stages of cytolysis, which appear in the lumina of some alveoli as large, darkly stained bodies with obscure nuclei. Also, very numerous spherical droplets of lipoids and other products of cytolysis are characteristic of individuals which have completed a period of ovogenesis and have spawned (or, when conditions were not conducive for spawning, have resorbed their gametes).

Since the reproductive process is cyclic, the spent phase merges with and overlaps somewhat the inactive phase. Sharp distinction between the two is a convenience rather than a natural phenomenon.

Male gonads

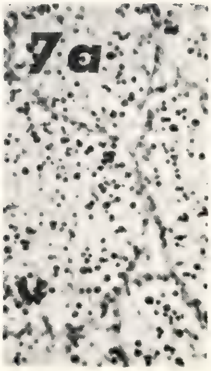
The criteria for the five corresponding categories of gonad condition in the male are presented below.

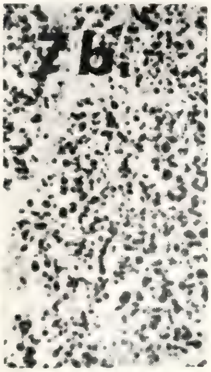
1. *Inactive phase*

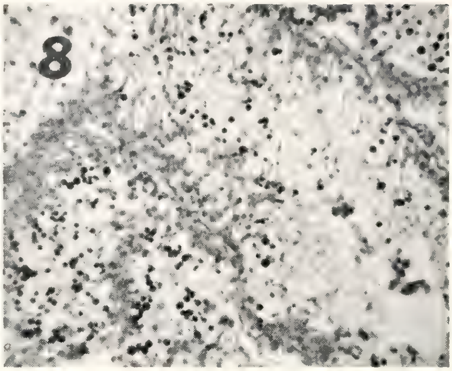
During the inactive phase, the male tissues frequently contain the products of an aberrant meiotic activity, which Coe and Turner (1938) have called "atypical spermatogenesis." Pycnotic cells (Fig. 7a) and multinucleated, non-pycnotic cysts (Fig. 7b) appear in the follicle cells. Many tissues contain both types although one usually predominates over the other. Both types of inclusions disappear during normal spermatogenesis, but reappear before it ceases, as do the nutritive granules seen in female tissues. Follicle cells fill the alveoli, surround the aberrant cells, and imbed the few spermatogonia and primary spermatocytes at the periphery of alveoli.

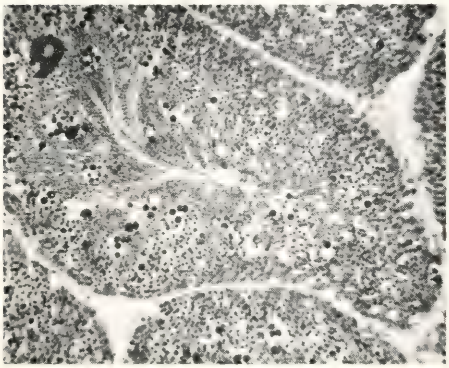
2. *Active phase*

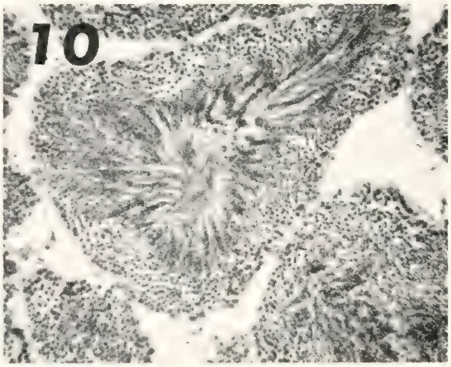
The active phase of the reproductive cycle in the male includes the entire process of spermatogenesis, unlike that in the female where only pre-meiotic stages are included. At the onset of the active phase, tissue sections contain proliferating primary spermatocytes at the basal membrane of the alveoli (Fig. 8). These small and uniformly sized cells, very similar in appearance to the earliest ovocytes, force their way between follicle cells and toward the centers of alveoli. Early stages of meiosis occur in the spermatogenic cells near the basal membrane, whereas the

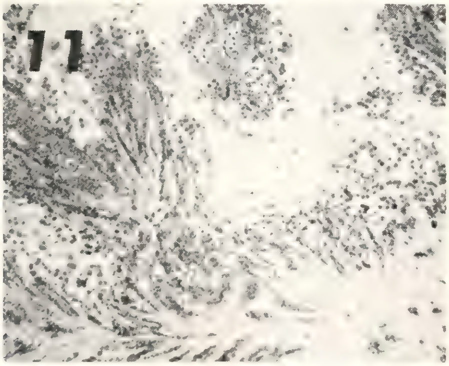












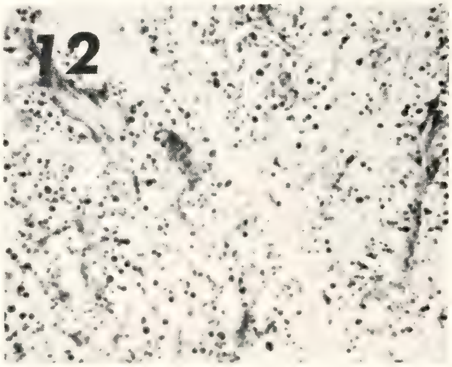


FIGURE 7a. Section of gonad tissue from male clam in inactive phase of reproductive cycle. Inactive phase of spermatogenesis in male soft-shell clams. Note the round, pycnotic cells, products of atypical spermatogenesis.

FIGURE 7b. Same, showing the cysts of multinucleated, non-pycnotic cells, also products of atypical spermatogenesis.

FIGURE 8. Section of gonad tissue from male clam in active phase of reproductive cycle, showing early stages of spermatogenesis.

FIGURE 9. Same, showing later stages of spermatogenesis. Late, active phase of spermatogenesis.

later spermatids occur at the centers of alveoli (Fig. 9). A very thin-walled membrane surrounds each spermatid. Meiotically active cells between the periphery of the alveoli and their centers eventually obliterate the follicle cells. A large number of spermatids produce a distinct mass in the centers of alveoli.

3. *Ripe phase*

Ripe male tissues contain masses of spermatozoa, arranged in more or less radial columns with their tails oriented toward the center. In fully ripe specimens these spermatozoa may nearly fill the lumina of the alveoli (Fig. 10).

4. *Partially spawned phase*

Partially spawned male tissues contain relatively few spermatogonia at the basal membrane (Fig. 11). Follicle cells occur between the basal membrane and groups of cells undergoing spermatogenesis. A scattering of pycnotic cells occur within the follicle cells. Spermatozoa still occupy a substantial portion of the central alveolar area.

5. *Spent phase*

Spent male tissues contain no or very few spermatozoa in the central alveolar area (Fig. 12). Numerous follicle cells with multinucleated, non-pycnotic cysts and pycnotic cells from atypical spermatogenic activities surround small groups of spermatozoa. Spent tissues lack cells in the active phase of spermatogenesis.

Geographic Variations in the Reproductive Cycle

Eastern Maine

Clams in the inactive phase were encountered throughout the year in eastern Maine, but were least numerous during June (Fig. 13). All were in this condition from September through December in 1959 and 1960. By January, 1960, some had begun gametogenesis. Clams in the active phase became more numerous each month after January, until May, after which fewer individuals were in this condition, and were absent from the August samples. Clams in the active phase were obtained to some extent for a period of about seven months.

Ripe clams were first observed in the middle of May, but none showed indications of having spawned. In June about half of the clams were ripe and some were partially spawned. Partially spawned clams were more numerous in July and by August about 75% of the clams had completely spawned and had returned to the inactive condition. The spawning period, then, extended from early June to about the middle of August, a period of 2½ months.

Minor exceptions to the reproductive cycle described above were observed in clams from two eastern Maine areas where gametogenesis began earlier than in the

FIGURE 10. Section of gonad tissue from ripe male clam. Note the columns of spermatozoa.

FIGURE 11. Section of gonad tissue from male clam in partially spawned phase.

FIGURE 12. Section of gonad tissue from spent male clam. Note the occurrence of both types of atypical spermatogenesis.

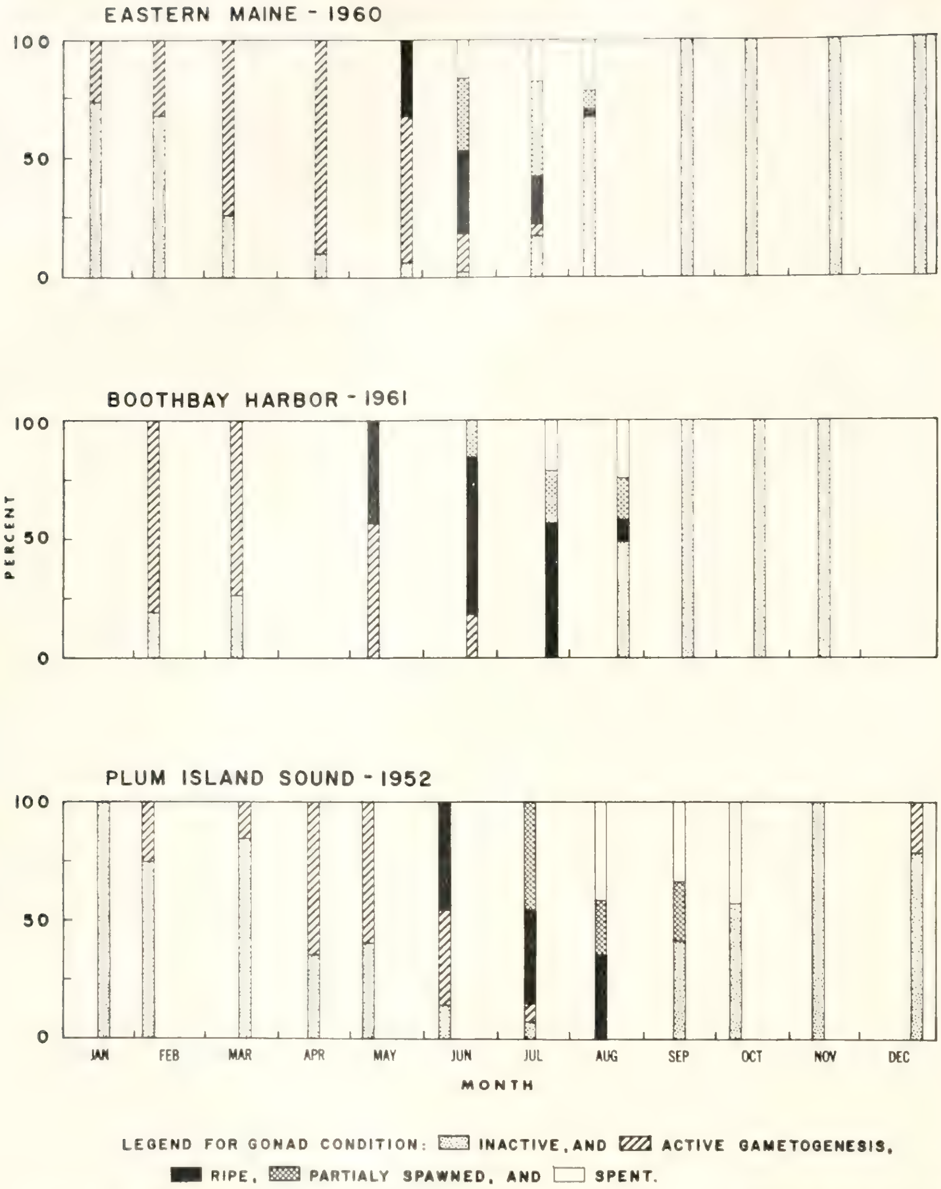


FIGURE 13. Gonad conditions of *Mya arenaria* from New England. The length of each shaded area represents the percentage frequency of clams in each gonad condition.

five other areas. More than half of the Lubec and Pembroke clams were in the active phase in January and February. After February, clams in this condition were almost equally as numerous from all seven eastern Maine areas. An earlier spawning in Lubec and Pembroke was not definitely indicated by the data.

Boothbay Harbor

Gametogenesis had commenced in about 75% of Boothbay Harbor clams during February and March (Fig. 13). More ripe clams occurred during May in the Boothbay Harbor than eastern Maine samples. Some partially spawned clams were observed as early as June in Boothbay Harbor, but both areas yielded numerous partially spawned clams during July. Completely spawned clams did not appear until July in Boothbay Harbor, or nearly one month later than in eastern Maine. More Boothbay Harbor than eastern Maine clams were in the ripe, partially spawned or spent condition in late August. The three-month spawning period of Boothbay Harbor clams, extending from late June to August, was not only later, but longer by a half a month than that of eastern Maine clams.

Plum Island Sound

The spawning season was still later in clams from Plum Island Sound than in clams from all Maine study areas (Fig. 13). Relatively few clams began gametogenesis during February and March. Ripe clams appeared first in June, a month later than clams from Maine. Correspondingly, the earliest occurrence of spawned clams was later. Spawning, indicated by the appearance of partially spawned clams from July through September, lasted for three months in Plum Island Sound. Many spent clams were seen as late as October.

Discussion

The results of gonad examinations suggested several generalizations about the reproductive cycle of *Mya arenaria*. Gametogenesis began during the late winter or early spring in all areas sampled. Thereafter a progressive development of the gametes was seen until ripe cells filled the tissues and spawning began in late spring and early summer. A spawning peak occurred during the middle and late summer, after which gametogenesis ceased. The inactive condition prevailed during the fall and most of the winter. No more than a single reproductive cycle occurred in the clams from nine sample areas north of Cape Cod. No failure to spawn was indicated in clams from any of the sample areas.

Despite the more southern latitude, the reproductive period in Plum Island Sound was characterized by a later rather than an earlier spawning season than that in Maine (Fig. 13). Furthermore, a still later spawning season than that of 1952 was indicated by the occasional gonad samples taken in Plum Island Sound during the preceding year. A few ripe clams first appeared during July, 1951. Both ripe and partially spawned clams were most numerous during late July and early August. All were spent by late October, 1951.

Most reports of larval occurrence and sex cell development indicated that *Mya* begins spawning at an earlier date in areas north of Plum Island Sound (Fig. 14). At Malpeque Bay, Canada, the earliest soft-shell clam larvae were found during early June by Stafford (1912) and during late May by Sullivan (1948). Stafford (1912) found an abundance of *Mya* larvae during July and August at St. Andrews, Canada. Most of the clams in Battle's (1932) samples from St. Andrews, Canada, contained ripe gonads during early June. She found some spent gonads by late June, indicating the beginning of the spawning season.

After finding an early abundance of larvae, Welch (1953, unpublished report)[2] deduced that some were probably in the plankton of Robinhood Cove, Maine, during early May, 1951. He also caught a few larvae during June of 1952 (oral communication), even though maximum numbers occurred during the middle of August to the middle of October that year.

A late spawning season was reported for *Mya* in northern Massachusetts areas. Belding (1907) observed that *Mya* spawns from the middle of August until early October (Fig. 14). Stevenson (1907) caught no larvae at Ipswich, Massachusetts, before late August, and reported the last half of September as the period of peak spawning. He also caught large numbers of larvae at Plymouth, Massachusetts, during late July and early August. Later Belding (1930) reported July and August as the spawning season for soft-shell clams north of Boston. These reports agreed with our observations of a late active phase and spawning period of *Mya* in Plum Island Sound.

A study of Figure 14, summarizing available information on the reproductive cycle of *Mya*, shows clearly two important phenomena: (1) the tendency for clams to develop gametes and spawn progressively earlier in the season northward and southward from northern Massachusetts, and (2) the bimodal nature of spawning activity south of Cape Cod. It is not entirely certain from this information that the bimodality results from two distinct reproductive cycles. It may result merely from an interruption in the spawning activity during a single reproductive cycle. Lacking observations on gametogenesis in the clams from their study areas, Bumpus (1898), Mead and Barnes (1904), Belding (1907; 1930), Stevenson (1907), Nelson and Perkins (1931), Deevey (1948), and Landers (1954) neither described nor implied more than a single annual reproductive cycle to *Mya*, even though Mead and Barnes (1904), Belding (1907; 1930), and Stevenson (1907) found two distinct groups of newly settled juveniles, and Landers (1954) observed two distinct swarms of larvae. In addition, Coe and Turner (1938) and Rogers (1959) believed that *Mya* probably spawned more than once each year, but did not mention the possibility of more than a single annual reproductive cycle.

Nevertheless, some evidence is available from several studies which suggests two separate reproductive cycles per year in southern latitudes. The clams from southern areas were reported to begin spawning earlier in the year than those from northern Massachusetts areas (Fig. 14). Deevey (1948) found that *Mya* larvae were dominant in Tisbury Great Pond on Martha's Vineyard, Massachusetts, from late April to June. Landers (1954) obtained larvae as early as the middle of April in samples from Wickford Harbor, Rhode Island, whereas Pfitzenmeyer (1962) captured larvae in the plankton of Chesapeake Bay, Maryland, throughout May. More important, however, both Landers (1954) and Pfitzenmeyer (1962) caught no larvae during the middle of the summer, after a late spring to early summer larval swarm. This period of larval absence in the plankton corresponded to observations of gonads by Rogers (1959) and Pfitzenmeyer (1962) which were apparently in the inactive phase during the middle of the summer. Hence, the clams were unable to spawn because ripe sex cells were not present in the gonads.

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[2] Welch, W. R., 1953. Seasonal abundance of bivalve larvae in Robinhood Cove, Maine. Fourth Annual Conference on Clam Research, U. S. Fish and Wildlife Service, Clam Investigations, Boothbay Harbor, Maine. (Mimeographed report).

Further evidence of biannual spawning can be drawn from our own histological examinations of clams collected in the Woods Hole, Massachusetts, area.

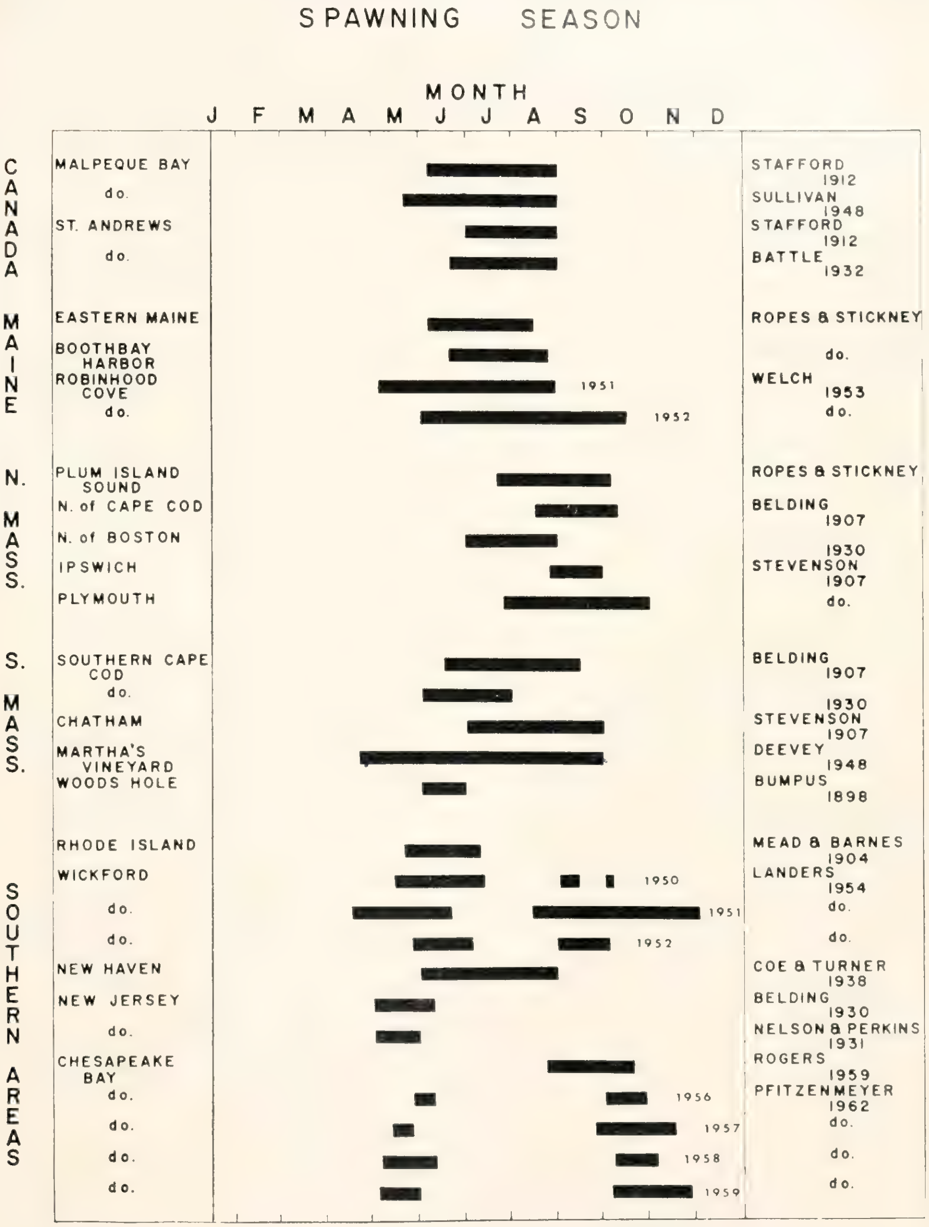

FIGURE 14. The duration of the spawning season of *Mya arenaria* reported in the literature for areas along the Northwestern Atlantic coast.

Samples of clams collected July 19 and 31, 1950, from Falmouth Inner Harbor contained none with ripe or developing gametes. Some live clams from these samples were held in outdoor tanks of sea water at seasonal temperatures until September 1, 1950, at which time most of them were fully ripe. The numerous nutritive granules, typically products of cytolyzed, unspawned gametes in females and the products of aberrant spermatogenesis in males, which were seen in July samples suggest that the clams from this population had also been ripe earlier in the year. This inference was supported by subsequent observations of ripe clams which were common in samples collected from Woods Hole both in late September, 1961, and in late May, 1962. The occurrence of ripe gonads during the early spring and fall corresponded to the times when larval swarms were found by both Landers (1954) and Pfitzenmeyer (1962). When the gonads of clams in the southern latitudes were in the inactive condition the gonads of clams in the northern latitudes were either ripe or partially spawned. Therefore, the two periods of gametogenesis in clams from southern areas were completely out of phase with the single annual reproductive period of northern clams, and indicated a biannual reproductive cycle.

We used clams with an apparent biannual reproductive cycle in spawning experiments at the Boothbay Harbor laboratory. Spawnings were induced during a period of a month or more after collecting clams from Woods Hole, Massachusetts, in late September, 1961, and also in late May, 1962, as well as from Chesapeake Bay, Maryland, in the middle of October, 1961. The Maryland clams remained in the inactive condition during the winter, and ripened again the following April in the laboratory tanks. These clams produced larvae when local clams were neither ripe enough to spawn, nor were the gametes developed to the ripe condition by the methods we tried.

We wish to thank Mr. Malcolm E. Richards, Marine Resources Scientist of the Maine Department of Sea and Shore Fisheries, for providing us with gonad samples from eastern Maine.

SUMMARY

1. Only a single reproductive cycle was observed in *Mya arenaria* gonads collected from areas north of Cape Cod.

2. In eastern Maine, the spawning season extended from early June to the middle of August. Boothbay Harbor clams spawned from late June to late August. Clams from Plum Island Sound began spawning in July, a month later than eastern Maine clams, and completed spawning by late September.

3. Observations of gonadal changes in *Mya* from southern Cape Cod indicated a biannual cycle occurs. These observations are supported by previous reports of spawning activities, larval occurrence, and gametogenesis in *Mya* from the southern latitudes.

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THE RELATIONSHIP OF ANTIGENIC COMPONENTS IN EGG–JELLIES OF VARIOUS AMPHIBIAN SPECIES [1, 2]

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In recent years, interactions between specific complementary surface substances, which are presumed to act in antigen-antibody-like fashion, have been postulated to account for a number of different kinds of specific intercellular reactions. Spiegel's investigation (1955) on the inhibition of reaggregation of dissociated embryonic cells and of dissociated sponge cells, tissue-incompatibility studies (Woerdeman, 1955), and tissue-affinity studies (Townes and Holtfreter, 1955) are examples of the type of cellular interaction which might be interpreted on the basis of complementary surface configurations.

Perhaps the most thoroughly investigated cells with reference to specific interacting substances are the spermatozoa and eggs of various marine invertebrates, particularly echinoderms. Studies of these substances have been given considerable attention in the past few years (for recent reviews, see Tyler, 1959; Metz, 1957, 1961; Runnström *et al.*, 1959). Evidence from several lines of investigation indicates that "fertilizin," the substance of the sea urchin egg-jelly, performs a significant if not essential role in fertilization (Tyler, 1955; Metz, 1957, 1961).

Likewise, several lines of study suggest that the jelly material surrounding amphibian eggs performs an essential function in fertilization. Thus, amphibian eggs without jelly, either as they normally occur in the body cavity or after removal of the jelly artificially, are not fertilizable (Bataillon, 1919; Kambara, 1953; Tchou-Su and Wang, 1956; Shaver and Barch, 1960; Subtelny and Bradt, 1961). Since jellyless body cavity eggs of the frog are capable of normal cleavage after artificial activation, either by inoculation of a cellular element (Bataillon, 1919), or after transfer of a blastula nucleus (Subtelny and Bradt, 1961), it is clear that the egg-jelly layer is not essential for development. It is reasonable to assume, then, that the jelly layer is involved in one or more essential interactions with the sperm in the process of normal fertilization. This assumption is supported by the observation that jellyless eggs can become fertilizable when artificially enrobed by jelly capsules taken from ovulated eggs (Subtelny and Bradt, 1961). Finally, Shaver and Barch (1960) have shown that antiserum prepared against the jelly-coat material of *Rana pipiens* will inhibit fertilizability of eggs of the same species. Since the jelly-coat material is immunologically tissue-specific (Shaver, Barch and Shivers, 1962) it appears that such inhibition of fertilization results from action of antiserum upon the egg-jelly material. In studies using nonprecipitating, univalent anti-egg-jelly sera, Shivers and Metz (1962) again obtained inhibition of fertilizing

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[2] A portion of this work was submitted as partial fulfillment for the degree of Doctor of Philosophy, Department of Zoology, Michigan State University.

capacity in frog eggs. These results with univalent antibodies indicated an actual blocking of egg-jelly receptor sites that perform some essential interaction with the sperm at fertilization. In view of this evident importance of the amphibian egg-jelly in fertilization, a more detailed description of this material seemed warranted. The present report shows that several antigenic components are present in amphibian egg-jellies. Some of these are common to several species, whereas others are restricted to a few or even a single species.

Materials and Methods

Jelly-coat material was mechanically removed with watchmaker forceps, subsequent to hydration in distilled water, from mature unfertilized eggs of four species of frogs (*R. pipiens, R. clamitans, R. sylvatica* and *R. catesbeiana*). The jelly was washed several times with distilled water and lyophilized until dry with a Virtis freeze-mobile. Standard antigen solutions for injection into rabbits were prepared by blending ten mg. of this lyophilate with one ml. of 0.85% sodium chloride, buffered at pH 7.4 with Sorenson's phosphate mixture, to which sodium ethyl mercurithio-salicylate (merthiolate, Lilly) was added in a proportion of one part per 10,000. Antigen solutions were also prepared, in the same manner, from egg-jelly capsules of species of Anura (*Bufo americanus* and *Bufo marinus*), as well as from another order of Amphibia (*Ambystoma maculatum*). An antigen preparation of fertilizin obtained by acid extraction (Tyler, 1956) from eggs of *Arbacia punctulata* (Echinodermata), which was kindly supplied by Dr. C. B. Metz, was also available.

Blood for control serum was drawn from the marginal ear vein of large albino rabbits (2.7 kilograms average weight) prior to the injection of antigen. No cross-reaction has been observed between normal rabbit serum and any of the amphibian egg-jelly solutions thus far tested. One and one half ml. of the standard antigen solution emulsified with an equal volume of Freund's complete adjuvant were injected *via* the subscapular route for the production of antisera. A booster injection consisting of the same amount of antigen and adjuvant was repeated 10 days later. A trial bleeding for the presence of antibodies was made three weeks after the second injection. If antibodies appeared at this time, bleedings from the ear vein were continued every other week for 6 to 8 weeks. Generally 3–5 rabbits were given injections with the same preparation and sera from these rabbits were pooled.

The antigenic components of the various jellies were analyzed by the agar-gel diffusion technique (Ouchterlony, 1949), slightly modified (Shaver, 1961). After agar plates were prepared in the usual way, various arrangements of wells were made, into each of which 0.75 ml. of the reactants was placed. The plates were developed for 5 days at room temperature (20–22° C.) and photographed for a permanent record.

In order to remove or neutralize specific antibodies the antisera were absorbed by mixing them with various dilutions of inhibiting antigen in glass tubes for 24 hours at 4° C. The antigen-antiserum mixture was centrifuged at 10,000 *g* to remove any precipitate which formed. The supernatant was then used as a test antiserum. Preliminary tests showed that an equal volume of the standard antigen preparation was sufficient to render serum non-precipitating. Unabsorbed antibody preparations were mixed with an equal volume of Sorenson's phosphate mixture before analyzing to maintain equal dilutions in the absorbed and unabsorbed antisera.

RESULTS

Analysis of antigens in species of Rana: Agar gel diffusion precipitin analysis of the antigenic components of egg-jellies of the four *Rana* species revealed similar patterns to the extent that the jelly of each species contained at least five distinct antigens. One of these antigens was common to all four species. Each of the species had a unique combination of cross-reacting antigens and two or three species-specific antigens. The relationships for anti-jelly serum of *R. pipiens* are illustrated in Figure 1. This figure is a drawing of a fully-developed agar diffusion plate in which unabsorbed anti-jelly serum of *R. pipiens* (Well PA) was reacted with antigens prepared from egg-jelly material of *R. sylvatica* (Well SJ), *R. pipiens* (Well PJ) and *R. clamitans* (Well CJ). Those components which are present in the jelly of eggs of *R. pipiens* are represented by lines formed between the antiserum well and well PJ (*e.g.,* lines a, b and c, Figure 1). The three lines labelled a are species-specific to *R. pipiens.* The curvature of the line nearest the antiserum well does not represent a cross-reaction between the antiserum and the heterologous jellies. The component common to the egg-jellies of all three species is indicated by the continuous line extending between the antiserum well and the three antigen wells (line b, Figure 1). Components present in the egg-jellies of *R. pipiens* and *R. clamitans,* but not present in the jelly of eggs of *R. sylvatica,* are represented by a continuous line between the antiserum well and wells CJ and PJ (*e.g.,* line c, Figure 1), but not present opposite well SJ.

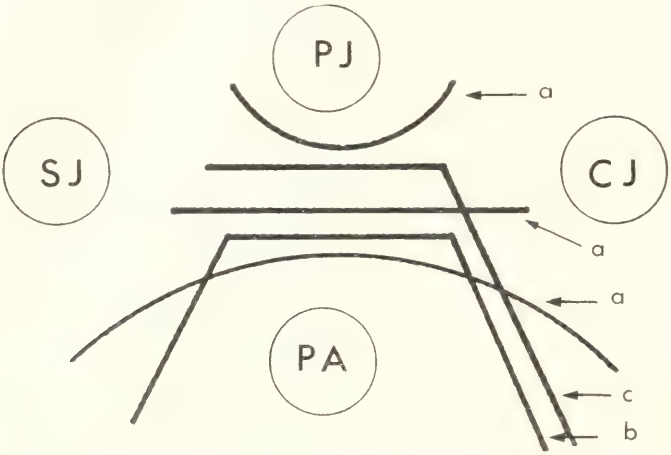

FIGURE 1. Diagram of double diffusion plate, showing precipitation bands formed by reacting anti-jelly serum of *R. pipiens* (PA) with the egg-jelly material of *R. sylvatica* (SJ), *R. pipiens* (PJ) and *R. clamitans* (CJ). a, b and c = precipitation bands representing components which are species-specific, shared among the three species and shared between only two of the species, respectively.

These relationships of jelly antigens were confirmed by appropriate absorption

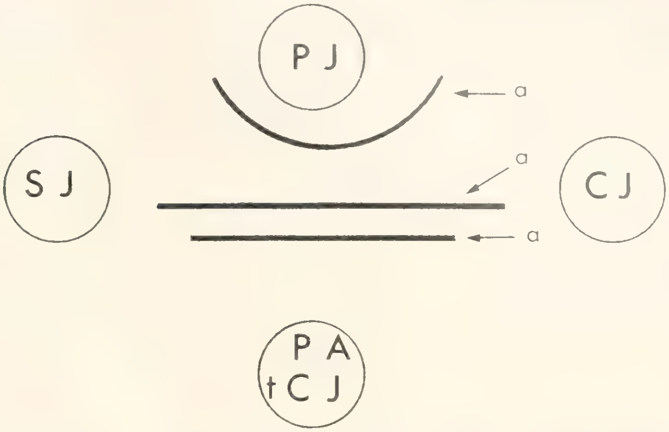

Figure 2. Diagram of double diffusion plate showing precipitation bands formed by reacting anti-jelly serum of *R. pipiens,* which had previously been absorbed with jelly of *R. clamitans* (PA+CJ), with jelly antigens shown in Figure 1. a = precipitation bands representing components which are species-specific.

of anti-jelly sera prior to the testing of these sera on agar-gel plates. As expected, antiserum failed to produce any precipitin lines following absorption with egg-jelly of the homologous species. In addition, antiserum prepared against the egg-jelly of one species was absorbed with jelly material from eggs of other species. Such absorbed serum was then diffused against egg-jelly solutions of several species (Figs. 2 and 3). Absence of precipitin lines between the absorbing heterologous jelly and the absorbed serum (*e.g.,* wells CJ and PA + CJ, Figure 2; wells SJ and PA + SJ, Figure 3) served as a check for complete absorption. The spectrum of lines between the homologous jelly and the absorbed serum represented those antigens not present in the absorbing heterologous jelly. Some of these repre-

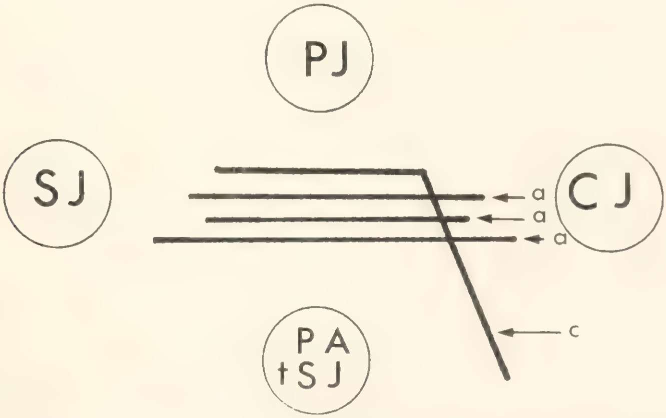

Figure 3. Diagram of double diffusion plate showing precipitation bands formed by reacting anti-jelly serum of *R. pipiens,* which had previously been absorbed with jelly of *R. sylvatica* (PA+SJ), with jelly antigens shown in Figure 1. a and c = precipitation bands representing components which are species-specific and shared between two of the species, respectively.

sented species-specific antigens since the lines appeared even after absorption and failed to join with lines of the other heterologous species (*e.g.*, lines a, Figures 2 and 3). Finally, joining of precipitin lines of the homologous and additional heterologous jellies indicated sharing of antigens not present in the original absorbing heterologous species (*e.g.*, line c, Figure 3).

By means of similar analysis employing antisera made against the egg-jellies of *R. clamitans, R. sylvatica* and *R. catesbeiana,* the relationships of the antigenic components in the egg-jellies of these species were ascertained. The results are diagrammed in Figure 4. The figure shows a component shared among all four species of *Rana*; a component shared among *R. pipiens* and *R. clamitans* and *R. catesbeiana* which is not present in the jelly of *R. sylvatica;* a component shared between *R. clamitans* and *R. catesbeiana* which is not present in jellies of *R. pipiens* or *R. sylvatica;* and a number of components which are species-specific in each case. A component common to *R. pipiens* and *R. sylvatica* could be detected by the anti-jelly serum of *R. sylvatica* but not by the *R. pipiens* antiserum.

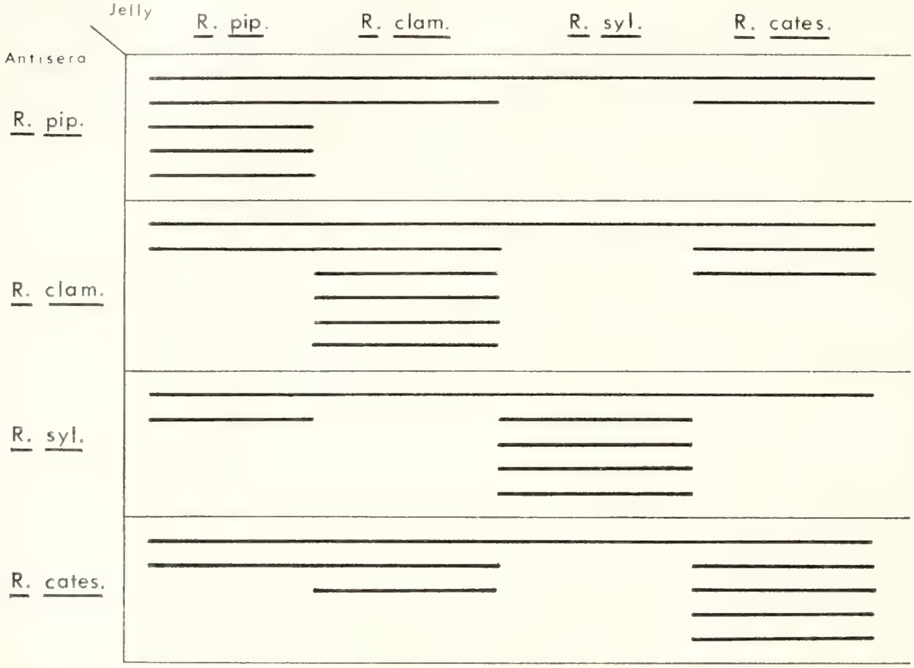

FIGURE 4. Interrelationships of antigens in egg-jellies of species of *Rana* as determined by agar-gel diffusion analysis. Antigens were determined by reacting the anti-jelly serum of each species with the various jellies. Common antigens are represented by continuous lines. Lines for each species represent a minimum number of antigenic components.

In addition to the interrelationships among the egg-jelly antigens of the four species of *Rana,* jellies of some more distantly related species were examined. These were *Bufo americanus, B. marinus, Ambystoma maculatum* and the echinoderm, *Arbacia punctulata.* These results are presented in Table I.

A number of antigenic components were observed which were specific to the jelly of *B. americanus,* as well as other antigens which were shared between this species and *B. marinus.* Cross-reactions were also observed between antiserum against *B. americanus* egg-jelly and the jellies from the four species of *Rana.* When reciprocal combinations of the jelly antigens of *B. americanus* and the anti-jelly serum of *A. maculatum, R. clamitans* and *R. catesbeiana* were made to react, no precipitin bands appeared.

Diffusion of *Bufo marinus* jelly against homologous antiserum resulted in at least five precipitin bands. Some represented specific antigens, whereas others represented antigens shared with *B. americanus.* In contrast to *B. americanus,* when *B. marinus* egg-jelly antiserum was reacted with jelly antigens from the species of *Rana,* no cross-reactions were observed.

TABLE I

Antigenic components in egg-jellies of species of Rana and Bufo (Anura), Ambystoma (Urodela) and Arbacia (Echinodermata; Echinoidea) as determined by agar-gel diffusion precipitin tests

Anti-egg-jelly serum of \ Jelly	*B. amer.*	*B. mar.*	*A. mac.*	*Arb. punc.*	*R. syl.*	*R. cates.*	*R. pip.*	*R. clam.*
Bufo americanus	5	3	2[a]	0	1	2[a]	2	2[a]
Bufo marinus	3	5	0	0	0	0	0	0
Ambystoma maculatum	0[b]	0	5	0	0[b]	2[a]	0	0
Arbacia punctulata	0	0	0	2	0	0	0	0

Numbers represent precipitin bands which appeared by reacting the anti-jelly serum with the jelly. a. reciprocal reaction produced no bands. b. reciprocal reaction produced two precipitin bands.

The antiserum prepared against the egg capsules of *Ambystoma maculatum* produced five precipitin bands when made to react with the homologous jelly antigen. When the antiserum of this urodele species was reacted with the jelly antigens of the four species of *Rana,* and of the two species of *Bufo,* only the jelly of *R. catesbeiana* produced bands. The reciprocal combination of jelly antigens of *A. maculatum* with the anti-jelly serum of *B. americanus* and *R. sylvatica* produced two precipitin bands.

The antiserum prepared against a sample of "fertilizin" made from jelly material of eggs of *Arbacia punctulata* produced two specific precipitin bands when reacted with the homologous jelly but gave no visible reactions with jelly antigens from any of the species of Amphibia.

DISCUSSION

The species-specificity of fertilization is well nigh an axiom of biology. This specificity, combined with the high correlation between cross-fertilization and cross-agglutination of spermatozoa by substances from eggs, especially in echinoderms (*cf.* Tyler, 1959, for earlier work on this topic), suggests that there are specific molecular patterns on the surface of gametes which interact during fertilization. In addition to the specificity factor of fertilization, other roles in fertilization have been attributed in part to the interaction of egg and sperm surface substances. These include attachment of sperm to the egg, initiation of acrosomal reaction, sperm engulfment and activation of the egg. The egg-jelly material of both echinoderms and amphibians has been suggested as one of the complementary egg surface substances which interacts with the sperm at fertilization. Additional evidence for the egg-jelly of amphibians being important in the initial steps in fertilization is the fact that antibodies prepared against these jelly antigens inhibit the fertilizability of eggs (Shaver and Barch, 1960; Shivers and Metz, 1962). These observations suggest that surface components in amphibian gametes may represent the same mechanism for the insurance of specificity of fertilization in this group that the "fertilizin-antifertilizin" system may represent in the echinoderms.

The specificity of fertilization in amphibians is not absolute, but allows a certain degree of cross-fertilization between species (for a summary of the variety of crosses which have been made among amphibians, see Moore, 1955). Crosses between species of *Rana* employed in the present study (*R. pipiens, R. clamitans, R. sylvatica* and *R. catesbeiana*) are capable of some development with the exception of crosses involving the ova of *R. clamitans*. It may be inferred that one of the factors in the successful union of gametes of different species would be the degree of similarity of configurations on the surface of gametes participating in cross-fertilization. Thus, if the jelly-coat material of the amphibian egg plays a role in the specificity of fertilization one would expect to find similar substances in the jelly of eggs of species capable of hybridization. It should be noted that where cross-fertilization has been reported to occur between species of *Rana* whose egg-jelly antigens were analyzed in this study, common antigenic components were found to be present. In addition, common antigenic components were found to be present in the egg-jelly of some species which have not been reported as being capable of cross-fertilization (*e.g.,* an antigenic component shared between the egg-jelly of *R. clamitans* and the heterologous species of *Rana*). It is possible that the sperm of these species are capable of making contact with and penetrating the surface of the ova of *R. clamitans* without the subsequent rotation or further development of the egg.

The reasons for the differences observed between reciprocal reactions (*e.g.,* an antigen common to *R. sylvatica* and *R. pipiens* which could be detected by *R. sylvatica* antiserum but not by *R. pipiens* antiserum) cannot be determined from present experiments.

The species-specificity of fertilization may be of a different order of sensitivity than the identities or similarities of antigenic components as determined by precipitation analysis. The similarity of a jelly antigen between two or more species as determined by immunological procedures may not necessarily imply that cross-fertilization would result from approximating gametes of these species. On the other hand, if these egg-jelly antigens perform a significant role in fertilization, then antibodies against them should combine with and block them at the cell surface, thereby inhibiting fertilization. In other experiments (Shivers, 1961 and unpublished results) it was found by selective absorption of antisera that the fertilizability of eggs of *R. pipiens* was markedly inhibited by treatments with two classes of antibodies: (1) those prepared against the species-specific components of *R. pipiens*; and (2) those prepared against the common component of all four species of *Rana*. That the species-specific components are the ones most likely to be operative in the initial steps of fertilization is deducible from the fact that the fertilizability of eggs was inhibited most strongly by antibodies against the species-specific components; and these are probably the last jelly components to be laid down on the egg during its sojourn in the oviduct (Barch and Shaver, 1963). As expected, the antibodies against the species-specific components of heterologous egg-jellies (*R. clamitans* and *R. sylvatica*) had no effect on the fertilizability of eggs of *R. pipiens*.

Although much remains to be done to elucidate the role of frog egg-jelly capsules in fertilization, these observations on the interrelationships of antigenic components in the jellies and the effect of antibodies against them on the fertilization reaction offer more evidence for an essential role(s) for the jelly-coat material.

The author wishes to express his thanks to Drs. John R. Shaver and S. H. Barch for their many suggestions during the course of this work. Thanks are also due Dr. C. B. Metz for reading the manuscript.

SUMMARY

1. Antisera were prepared against the jelly-coat material of eggs of several species of *Rana* (*R. pipiens, R. clamitans, R. sylvatica* and *R. catesbeiana*) and other species of Amphibia (*Bufo americanus, Bufo marinus* and *Ambystoma maculatum*). Serological characterizations as to species-specificity of antigenic components found in these jellies have been presented.

2. Analysis showed that the jellies of each species contained a number of species-specific components.

3. Common components were observed in the jelly of species belonging to the same genus (either *Rana* or *Bufo*).

4. In certain cases common components were observed between species of different genera (*Bufo americanus* and each of the species of *Rana*).

5. The results of these studies on the specificity of antigenic components in egg-jellies are discussed in connection with the possible role of these components in the process of normal fertilization.

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AN AUTORADIOGRAPHIC STUDY OF THE RELATION BETWEEN HEMOCYTES AND CONNECTIVE TISSUE IN THE WAX MOTH, GALLERIA MELLONELLA L.[1]

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Considerable uncertainty exists as to the origin of the connective tissue sheaths around insect organs and the possible participation of blood cells in the development of connective tissue. Lazarenko (1925) made the first significant study of connective tissue in insects, and suggested that hemocytes participate in its formation. Wermel (1938) agreed with this, and so did Wigglesworth (1956) though the latter author considered the connective tissue sheath around the central nervous system, the neural lamella, a special case. Wigglesworth thought that the neural lamella of *Rhodnius* was secreted by an underlying layer of cells, the sheath cells, but that hemocytes probably contributed mucopolysaccharide to it. In contrast, Scharrer (1939) thought that the neural lamella of cockroaches was secreted entirely by the underlying sheath cells, and Bonhag and Arnold (1961) thought likewise for the connective tissue sheath around ovarioles. In moth pupae the larval connective tissue sheaths are destroyed, the organs then reorganized into their adult form, and then a new connective tissue sheath is formed. Ashhurst and Richards (1964a) concluded that one type of hemocyte (the adipohemocytes) participates in destruction of the larval neural lamella in moth pupae, but that hemocytes play no role in the later formation of the adult neural lamella around the nerve cord. All of the above studies were based on subjective deductions from histological appearances. The present study represents an attempt to obtain unambiguous objective data for the neural lamella in moth pupae, using a radioautographic technique. Support was obtained for the conclusions of Ashhurst and Richards.

MATERIALS AND METHODS

Larvae of the wax moth, *Galleria mellonella* L., were reared on an artificial diet (Haydak, 1940) at 39° C. and 75% R.H. in darkness.

Hemocyte counts (HC) were made by chilling animals in a refrigerator to deter blood coagulation, removing 2 μl. of blood, diluting it 70 × in a quick-diluting micropipette which could be twirled for mixing (Fig. 1), and then counting in a standard Levy hemocytometer. To obtain blood, incisions were made on the mid-dorsal line of larvae, in the lateral thoracic wall of pupae. The 2-μl. sample

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was taken from the third drop of blood that exuded. The stated values are averages of determinations from nine different individuals.

Hemocytes were labeled with tritiated thymidine obtained from Schwarz Bio-Research Inc. The samples used had specific activities of 1.9 c./mM = 0.50 mc./ml. Injections of 5 μl. (= 2.5 μc.) were used with larvae and pupae of approximately 200 mg. live weight. In some cases, two or even three injections were made at successive intervals to increase the percentage of labeled cells.

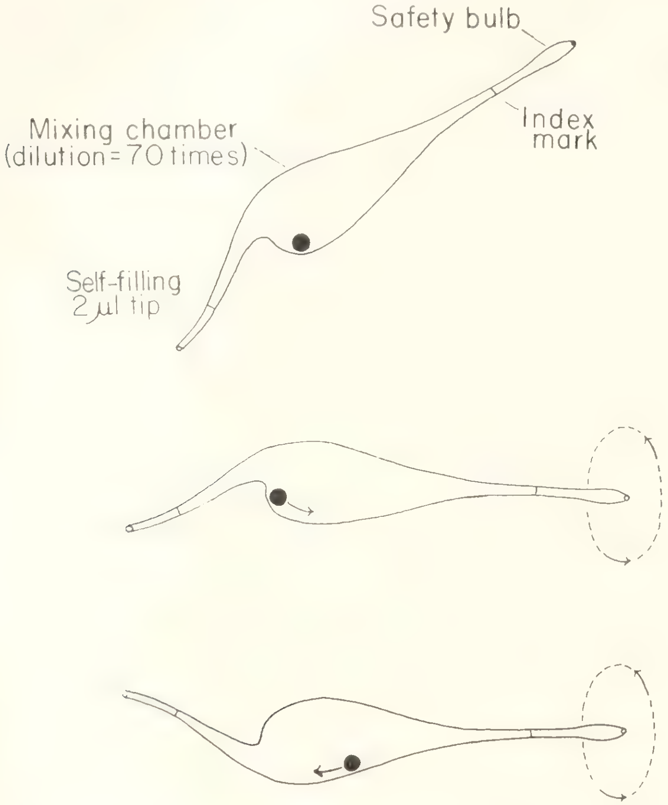

FIGURE 1. Diagrams showing the quick-diluting micropipette (2 μl.) and method of using it.

Blood transfusions from labeled prepupae and pupae to other prepupae and pupae of the same age were made in a cool room (15° C.) six hours after injection of H^3 thymidine into the donor. The receiving prepupae and pupae were bled as much as feasible (40–60 μl.) and then given a corresponding amount of blood from the labeled donor. The transfused animals were returned to a 35° cabinet to continue development.

Parabiotic twins were made using a labeled and a normal pupa of the same age. A few crystals of phenylthiourea, streptomycin sulfate and K penicillin were placed around the wound site before sealing the partners together with beeswax. After the operation, the pupae were pumped alternately with finger pressure to mix the two blood streams and to assure free flow of blood with hemocytes from the donor to the pupa receiving labeled hemocytes.

The fate of labeled hemocytes was checked by examination of serial sections. At intervals of 18, 72 and 92 hours after pupation, pupae were killed by injection of Zenker's fluid into the thorax. The insects were then opened and fixed for 18–20 hours, washed overnight, dehydrated through an ethanol series, embedded in Tropical Ester Wax, and sectioned. Alternate sections from the ribbon were mounted on two sets of slides. One set was stained with Mallory's triple stain, the other set was used for autoradiography.

Autoradiographs were prepared by the method of Joftes (1959), using Nuclear Emulsion type NTB3 and exposing for 2–3 weeks. Unincorporated label, RNA, etc. were removed prior to autoradiography by treating the sections with 10% perchloric acid for two hours at 20° (Woods and Taylor, 1959).

RESULTS

Prior to using autoradiography to study the role of hemocytes in connective tissue formation it is necessary to obtain labeled hemocytes. Using tritiated thymidine it is possible to label nuclei permanently but only during mitosis. As a prelude, then, it is necessary to know when mitoses occur in hemocytes, to verify that mitoses can be stimulated to occur, and to learn something of the life cycle and life expectancy of hemocytes.

Normal hemocyte counts and the effects of bleeding

Stephens (1963) has recorded the mean hemocyte concentration or hemocyte count per mm.^{3} of blood (HC) in normal larvae of *G. mellonella* as 33,200 ± 1200. In insects in general the HC can change with age and stage due to an actual change in total hemocyte number, or the amount of plasma, or the number of cells adhering to the surfaces of various organs (Jones, 1962; Wheeler, 1963).

Our values for HC in *G. mellonella* were considerably lower than those given by Stephens but this may be due to the determinations having been made at a later time in development and utilizing the third drop of blood to exude from chilled individuals. At the end of larval development, when the larva has finished spinning its cocoon, the HC was found to average 15,000/mm^3. The value fell to 4000 in the motionless pharate pupa which is about to shed the larval skin, then rose gradually to regain the 15,000/mm.^{3} value about 12 hours later. The value then again decreased to about half this after another 12 hours (Figs. 2–3). How much of these changes may be due to each of the possible factors listed in the preceding paragraph was not determined; the values stated here represent only hemocyte concentrations in the circulating blood of chilled specimens.

Many years ago Dakhnoff (1938) reported that bleeding a larva of *G. mellonella* resulted in the production of excessive numbers of mitoses. Since it is only at mitosis that H^3 thymidine is incorporated as a permanent label in nuclei, the report suggests a method for obtaining a reasonable percentage of labeled hemocytes for use in transfusions and parabiotic twins. But we needed not only to verify Dakhnoff's report but also to see if the same increase in hemocyte count occurred during prepupal and early pupal stages. For this purpose late larvae and early pupae were bled either a small drop (*ca.* 1 μl.) or a large drop (*ca.* 5 μl.), and the HC determined after various intervals of time in comparison to HC values of normal individuals. As is shown by the curves in Figures 2 and 3, the bleeding resulted in an approximate doubling of the HC in the hemolymph. The time required for this doubling was longer in the pupa than in the late larva but both showed the phenomenon. And in both there was a stimulation of mitotic activity.

Bleeding did cause a delay in the pupation of larvae and in the development of pupae but this, being known, does not interfere with the tests to be reported in subsequent parts of this paper.

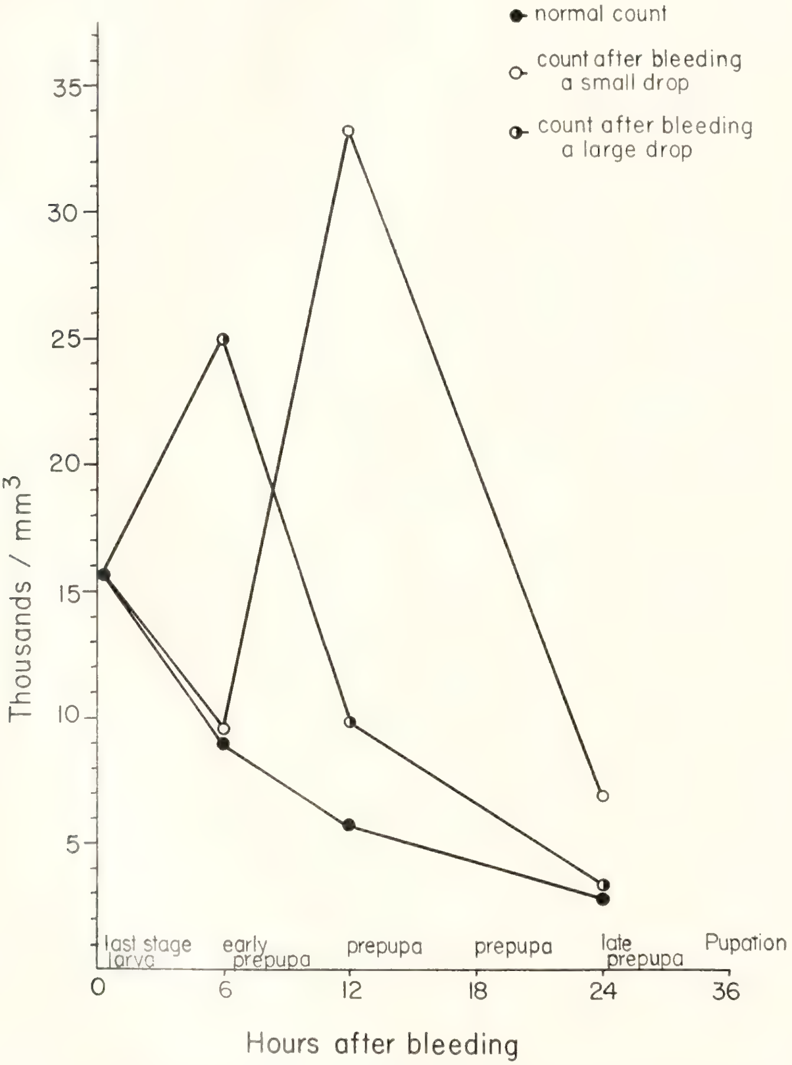

FIGURE 2. Curves showing the hemocyte concentration of larvae of *Galleria mellonella:* normal and after bleeding small and large drops.

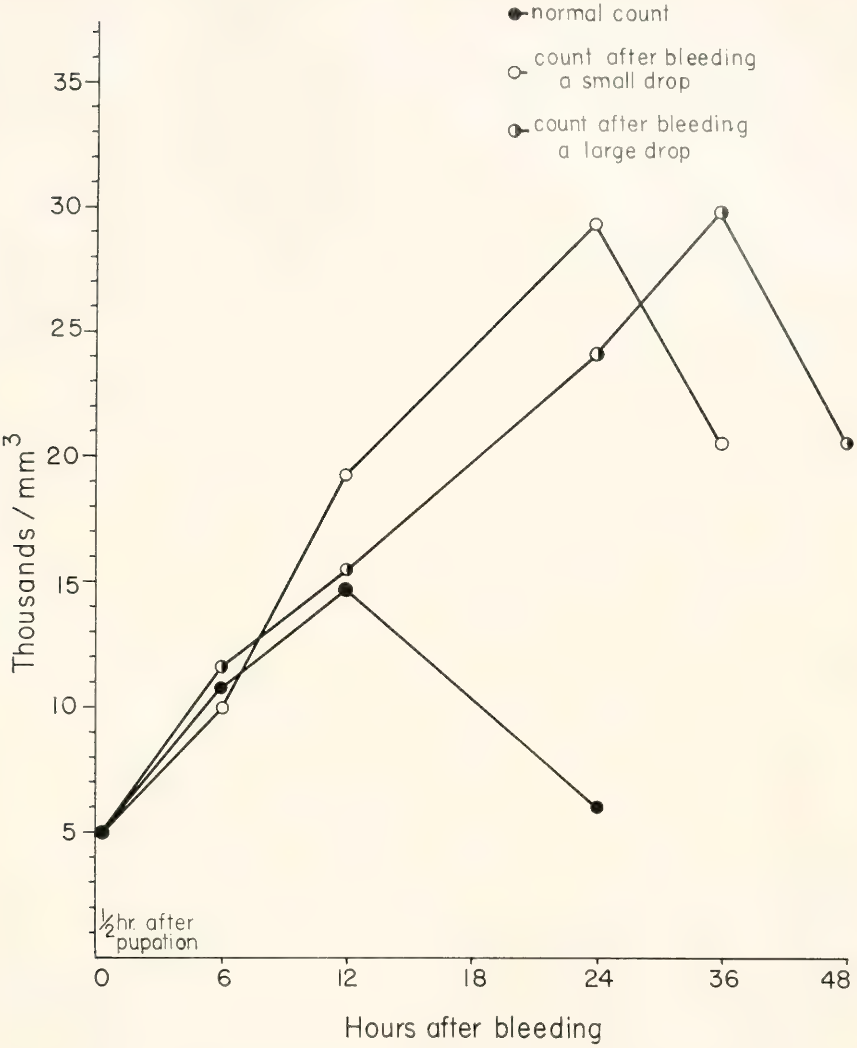

FIG. 3. Curves showing the hemocyte concentration of pupae of *Galleria mellonella*: normal and after bleeding small and large drops.

The conclusion from the above tests was to bleed larvae or pupae, then immediately inject H^3 thymidine. After 6–24 hours, depending on the amount of bleeding and the age of the individuals, blood could then be used for transfusion or individuals joined in parabiosis.

The life cycle and life expectancy of hemocytes

Dakhnoff (1938) attempted to trace the transformation of regenerating blood cells (induced by bleeding) of *G. mellonella* by comparing differential HC counts taken every hour. He arrived at a transformation time of an hour or so. But, as is evident, such curves could give only the speed of change in the differential hemocyte picture, not the actual time required for change. His method would not prove with certainty anything about the course of differentiation of individual hemocyte types.

To obtain definitive evidence, last instar larvae were bled a large drop (*ca.* 5 μl.) and then injected with 5 μl. of H^3 thymidine. Blood smears were made at repeated intervals, different specimens being used for each interval to avoid any complications that might arise from repeated bleeding of individuals. The blood films obtained were fixed with ethanol, dried, and autoradiographed. The final slides, after development, were examined by phase contrast microscopy without staining.

The hemocyte picture varies greatly from one group of insects to another. In *G. mellonella* there are five distinct types of hemocytes: prohemocytes, plasmatocytes, adipohemocytes, spherule cells and oenocytoids (photomicrographs given in Ashhurst and Richards, 1964b). It is generally thought that prohemocytes differentiate into the other types, or at least into plasmatocytes which may later develop fat droplets to become adipohemocytes. In the present study the following data were obtained:

Time after injection of H^3 thymidine	Picture of labeling
6 hours	*ca.* 10% of prohemocytes labeled
12 hours	some of labeled prohemocytes beginning to differentiate into plasmatocytes
24 hours	numerous plasmatocytes labeled, some beginning to develop fat droplets, few labeled prohemocytes
48 hours	both labeled plasmatocytes and labeled adipohemocytes present
72 hours	only adipohemocytes labeled
	(Pupation at this time)
96 hours	only adipohemocytes labeled
120 hours	only a few labeled adipohemocytes present
144 hours	no labeled hemocytes of any type

The above data are interpreted as showing that prohemocytes become labeled promptly, and that some of these soon differentiate into plasmatocytes which, with the development of numerous sudanophilic vacuoles, become adipohemocytes. In three days the sequence prohemocyte → plasmatocyte → adipohemocyte has been completed, and within six days the labeled adipohemocytes have vanished. It follows that the life expectancy of this cell type, at least at this stage in the life cycle of *G. mellonella,* is less than six days at 35° C.

The picture of blood cell differentiation is complicated by changes in hemocyte concentration treated in the preceding section and by changes in differential cell counts. All of the above cell types are found in late larval and early pupal life, but adipohemocytes become more numerous and more conspicuous after pupation. These changes, however, do not interfere with the use of labeled cells as presented in the next section.

Two other less common types of hemocytes are found in the blood of *G. mellonella.* These are the spherule cells and the oenocytoids. These two types

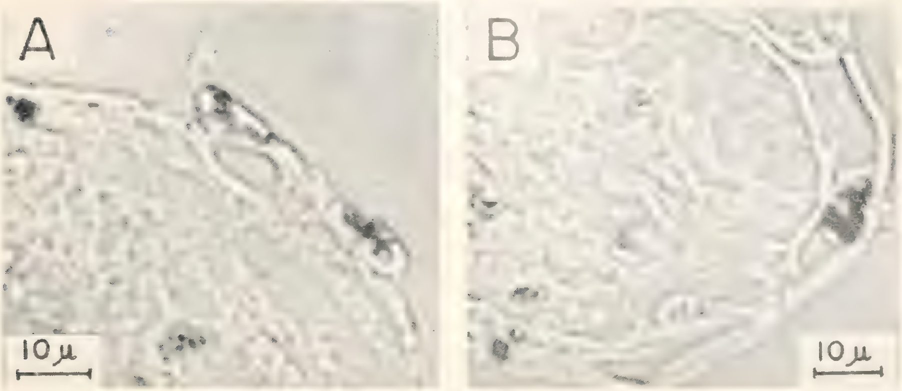

FIGURE 4. A. Autoradiograph showing labeled hemocytes in contact with the outer surface of the neural lamella of the nerve cord of *Galleria mellonella* 18 hours after pupation. B. Autoradiograph showing labeled hemocytes between laminae of the disintegrating neural lamella of the nerve cord of *Galleria mellonella* at about 18 hours after pupation.

are extremely difficult to identify with surety in our autoradiographed smears; hence nothing will be said about their origin or fate.

The relationship between hemocytes and connective tissue

The neural lamella around the central nervous system breaks down during the period 12–24 hours after pupation (Ashhurst and Richards, 1964a). Sections were prepared from 18-hour pupae which had received transfusions of labeled blood 6–48 hours previously. Labeled hemocytes were found throughout the hemocoel; some of these are appressed to the nerve cord prior to dissolution of

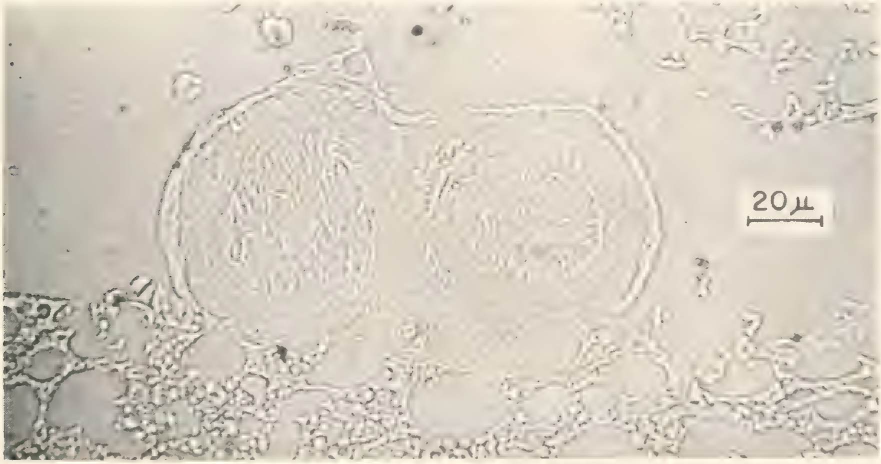

FIGURE 5. Autoradiograph of the abdominal nerve cord of *Galleria mellonella* 72 hours after pupation. This individual had been transfused with labeled blood. It shows the forming adult neural lamella without any label or labeled cells.

the neural lamella (Fig. 4 A), and, at a somewhat later stage, are found within the disintegrating laminae of the neural lamella (Fig. 4 B).

During the period 24–60 hours after pupation there is no neural lamella around the nerve cord (the central nervous system becomes reorganized to the adult condition during this time). A new neural lamella for the adult then begins to be formed and is clearly distinct by 72 hours (Ashhurst and Richards, 1964a). Sections prepared from 72-hour pupae which had been transfused with labeled blood or joined in parabiosis with a labeled pupa showed some labeled hemocytes in the hemocoel or appressed to various organs (especially fat body and tracheae) but no label in the sheath cells which are thought to secrete the neural lamella and no label in the neural lamella or immediately adjacent tissues (Fig. 5).

These data support the conclusion of Ashhurst and Richards that adipohemocytes have some intimate role in the destruction of the larval neural lamella during early pupal stages, but that neither these nor other hemocytes participate in the formation of the connective tissue around the adult nerve cord.

Summary

1. The hemocyte count of *Galleria mellonella* during the prepupal and early pupal stages varies from $4000/\text{mm.}^3$ to $15000/\text{m}^3$ as a function of age. The number of freely circulating cells can be approximately doubled within 6–24 hours by bleeding the insect.

2. Using tritiated thymidine and autoradiography it was found that there is a hemocyte differentiation sequence of prohemocyte → plasmatocyte → adipohemocyte. This differentiation requires about three days at 35°, and the labeled cells have disappeared in another three days. The life expectancy of these cells, at least in prepupal and early pupal stages, is then less than six days.

3. Transfusion of labeled hemocytes, followed by autoradiography of sections, confirmed the previous suggestion that adipohemocytes have some role in the destruction of the larval neural lamella, but that no blood cells are involved in the formation of the connective tissue around the adult nervous system.

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DURATION OF AN AFTER-EFFECT IN PLANARIANS FOLLOWING A REVERSED HORIZONTAL MAGNETIC VECTOR[1]

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Responsiveness of the living system to very weak magnetic fields is well established. Studies have involved organisms ranging from the unicellular *Paramecium* (Brown, 1962), through *Volvox* (Palmer, 1963), *Dugesia* (Brown, 1962), *Nassarius* (Brown, Brett, Bennett and Barnwell, 1960; Brown, Webb and Brett, 1960; Brown, Bennett and Webb, 1960; Brown, Webb and Barnwell, 1964), *Drosophila* (Picton, 1964), and Cockchafers (Schneider, 1963), to birds (Eldarov and Kholodov, 1964). The nature and strength of the response have been found to vary as functions of such factors as orientation of the experimental horizontal magnetic vector, of geographic orientation of the organisms, and of phase angles of the natural solar and lunar cycles.

It was recently demonstrated, furthermore, that the mud-snail, *Nassarius,* is able to distinguish strength differences of the horizontal magnetic vector at least within the range, 0.05 to 10.0 gauss, displaying a maximum in capacity to respond to an experimentally reversed horizontal magnetic vector when the directional change is effected with minimal simultaneous change in vector strength (Brown, Barnwell and Webb, 1964). Following subjection to horizontal vector fields differing from the earth's 0.17-gauss local one, the snails, upon return to the natural field, continued to display an influence of the experimentally imposed field. The effect lasted for at least three to five minutes. An after-effect of experimental magnetic fields, with strikingly similar characteristics, was discovered also for the planarian, *Dugesia* (Brown, 1965). An after-effect has also been reported for birds (Eldarov and Kholodov, 1964).

The present study was directed toward learning how long the after-effect, which results from an experimentally reversed magnetic field, persists beyond the previously demonstrated short period, and to learn something concerning the characteristics of its decay. More knowledge concerning these characteristics is of

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fundamental importance for the ultimate formulation of a sound hypothesis for the nature of the magnetoreceptive mechanism.

Materials, Methods and Experiments

Planarians, *Dugesia dorotocephala,* were observed as they moved initially northward over a polar-coordinate grid in an asymmetrical three-light field (Brown, 1962) during about a seven-month period from October 8, 1963, through April 30, 1964. The experimental conditions were designed to reveal the duration of the after-effect following response to a reversed magnetic field. Experimental series were usually run in pairs. Each of the two observers involved in a double series recorded planarian paths as follows. Five worms were placed in a 9-cm. Petri dish of water inside an orientation chamber where they remained for the duration of a 50-minute series. First, two 5-minute samples of worm-paths, to serve as controls, were recorded under the natural ambient environmental conditions. Thereupon, an 18-cm., cylindrical, alnico bar magnet was placed horizontally, centered directly beneath the point of initiation of the worm path, oriented to oppose the direction of the earth's horizontal vector, and located at such a distance below the orienting worms as to produce at the level of the worms a reversed horizontal field of the desired strength. Immediately thereafter the mean rate of turning of all the worms orienting during the first, the second, and the third five-minute interval was determined separately. The experimental magnetic field was then removed and the mean path for all the orienting worms was determined for each of the subsequent five-minute intervals during a 25-minute period.

One observer dealt with a reversed magnetic field of 4.0 gauss; another observer used a reversed field of 0.05 gauss. During the course of the 7-month experiment a total of five different observers participated and all observers dealt with both magnetic field strengths.

The observations were made at various hours of the day between 9 AM and 5 PM. About 60% were made during the morning hours. The total number of series run for each of the two magnetic field strengths for each of the seven calendar months was as follows:

	4.0 gauss	0.05 gauss
October	57	61
November	78	79
December	47	45
January	67	64
February	43	48
March	28	26
April	37	37
Total	357	360

It was learned later that the actual average number of worm-paths that was obtained in a 5-minute interval was about 14. The number varied from day to day depending upon the degree of activity of the animals. It is evident from the foregoing that during the 7-month period of the investigation there were about 10,000 initial control paths, about 15,000 paths during the 15-minute period of the applied fields, and about 25,000 paths during the 25 minutes following removal of the experimental fields.

The data were reduced in the following manner. For each of the single 50-minute series, the mean paths of the worms during each of the three 5-minute intervals while in the experimental field and during each of the five 5-minute intervals after removal of the field were expressed as differences from the average path determined for the initial two control samples in the same series. Mean values obtained in this manner for the "responses" to each of the two field strengths were then determined for all the data for successive 5-calendar-day periods. In addition, the mean was computed for each of the eight temporally corresponding differences between responses to the two experimental magnetic field strengths. Analyses of this report were based upon the eight values for each of the 5-day periods for the two experimental field strengths. This yielded a total of 656 individual data, or 328 differences.

It was quite conceivable that the worms would adapt to each of the two altered experimental fields in the course of time, and that the initial kind of turning behavior might gradually be restored despite continued presence of the experimental, reversed magnetic fields. To establish or eliminate this possibility, an additional experiment was performed. This second experiment was conducted between October 27 and December 22, 1964. It was designed just like the initial one except that the magnets were left in place during the whole 40 minutes instead of being removed after 15 minutes. It differed in addition, however, in the following ways; (1) Only two months were involved, overlapping in time of year, one year later, only about one-third of the period of the earlier experiment. (2) Six observers contributed to the results; four of them had not participated in the earlier observations and were given no information about either the earlier results or even the objectives of the study. (3) A larger fraction of the observations, about 65%, were made during afternoon hours. A total of 30 series was run at 0.05 gauss and 32 series at 4.0 gauss, distributed over the whole two-month period.

The data of this second experiment were treated like the earlier data in that responses were determined separately for each of the 5-minute periods by obtaining the path differences from those of the initial controls. The differences between the mean responses to each of the two field strengths for the corresponding consecutive time intervals were computed. The mean response to each strength for the first 15 minutes, the last 15 minutes and the intervening 10 minutes was calculated, together with standard errors of their means.

RESULTS

A. Removing magnets after 15 minutes

It is evident that if there were no influence of either of the two imposed reversed magnetic field strengths, no significant difference between the mean paths of the worms for the corresponding, successive pairs of 5-minute samples would be expected between the results for the two fields. If responses did occur, absence of significant difference between the two would indicate that exactly the same response resulted for the two strengths. Other than in magnetic-strength difference, the worms for the two kinds of series had been treated in precisely the same general manner by the same observers over a sufficiently long period to have reduced to insignificance the differences between the two means due to random sampling.

This method would also compensate for any systematic change that might tend to occur in the orientation of the worms over the course of the 50-minute observation period while in the orientation chamber. Such a systematic change could conceivably arise from a gradual photic adaptation, the repeated mechanical stimulation, or simply fatigue of the five worms. The procedure employed would be expected to eliminate, therefore, all changes which were independent of the specific differences between the effects of the two experimental magnetic treatments.

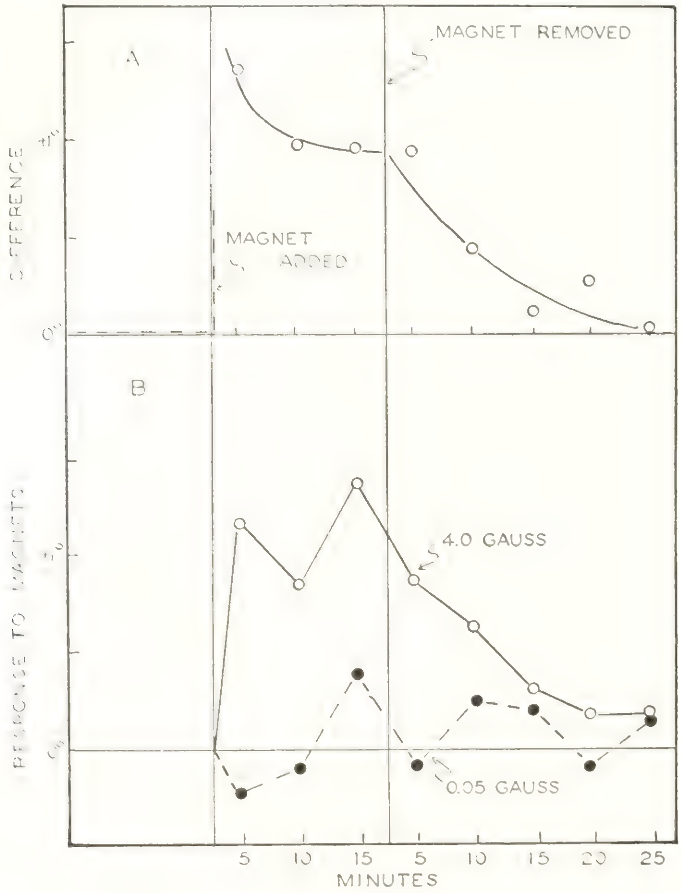

FIGURE 1. A, the response to a reversed 4-gauss horizontal magnetic field, expressed as difference from response to a reversed 0.05-gauss one, during three consecutive 5-minute intervals after application of the experimental fields, and during five 5-minute intervals following field removal. B, the responses to the two strengths of magnetic field, expressed as differences from initial controls, during and following the experimental field reversals.

A previous study (Brown, 1965) had established two facts: (1) north-directed worms in a 4.0-gauss reversed field turned more strongly clockwise than the same worms when they were subjected to a 0.05-gauss reversed field, and (2) during the initial 5-minute period following removal of the reversed field the worms that had been exposed to 4- to 6-gauss fields turned more strongly clockwise than the same worms subsequent to removal of 0.05- to 0.1-gauss fields.

In Figure 1 A, the mean path of the worms for each 5-minute interval in the 4.0-gauss series is plotted as the difference from the mean path for the corresponding interval in the 0.05-gauss series. Confirming the results of the previous study (Brown, 1965), worms in the 4.0-gauss field turned clockwise relative to worms in the 0.05-gauss one. There is suggestively present an initial overshoot and a subsequent tendency toward stabilization at a slightly decreased difference during the 15-minute period of exposure. Upon removal of the experimental, reversed fields, an after-effect persists with the same sign and of about the same magnitude during the first 5-minute interval. This is followed by a gradual decay of this after-effect with the difference between the after-effects from the two imposed experimental magnetic fields disappearing completely by the end of about 25 minutes.

It is seen from Figure 1 B, in which the mean responses of the worms to each of the two experimental field strengths, together with the post-field changes, are shown separately, that the results illustrated in Figure 1 A appear to come chiefly from a mean clockwise response to the 4.0-gauss field relative to essentially no mean response to the 0.05-gauss field.

B. Leaving magnets in place for 40 minutes

In Figure 2 A are seen the differences between responses of worms while in the reversed fields of 4.0 and 0.05 gauss for each of the eight consecutive 5-minute samples. In Figure 2 B the response to each of the field-strengths is shown separately. Although this figure is entirely comparable to Figure 1, it will be noted that the scale has been altered by a factor of 4. Both the responses of the worms to each field strength and the difference between the responses were much greater than for the earlier experimental series. In addition, a clear mean response was evident not only for the 4.0-gauss field (clockwise turning) but for the 0.05-gauss one (counterclockwise turning) as well.

One fact was immediately apparent from the results of this experiment, namely that there was no overall reduction in the influence of the experimental fields on the worms during the 40 minutes of exposure to these fields. First, considering grossly the differences between responses to the two field strengths, the difference was found for the first 15 minutes in the fields to be 3.87 ± 0.921° ($N = 186$) and for the last 15 minutes of the 40-minute exposure to be 3.97 ± 0.822° ($N = 186$.) Examining each field strength separately, for the 0.05-gauss field the first and last 15-minute groups of responses were −2.78 ±0.605° ($N = 90$) and −2.79 ±0.582° ($N = 90$), respectively. For the 4.0-gauss field, the comparable values were +1.09 ± 0.695° ($N = 96$) and +1.18 ± 0.581° ($N = 96$). The two mid-series values were +1.44 ±0.559° ($N = 62$) for the 4.0 gauss and −1.61 ±0.678° ($N = 60$) for 0.05-gauss.

It had been suggested, from examination of Figure 1, that there was an initial over-shoot in the response to the reversed fields with a stabilization at a slightly lower level prior to the removal of the experimental fields at the end of 15 minutes. A very similar behavior occurred again in this second experiment and, indeed, a partial apparent acclimation suggestively continued for about 20 minutes. Also as in the first series this behavior seemed to result principally from an alteration in response to the weaker, 0.05-gauss, field and only to a lesser extent to the 4.0-gauss one. But equally suggestive was an apparent complete loss of this partially "acclimated" state by the end of the 40-minute period of observation.

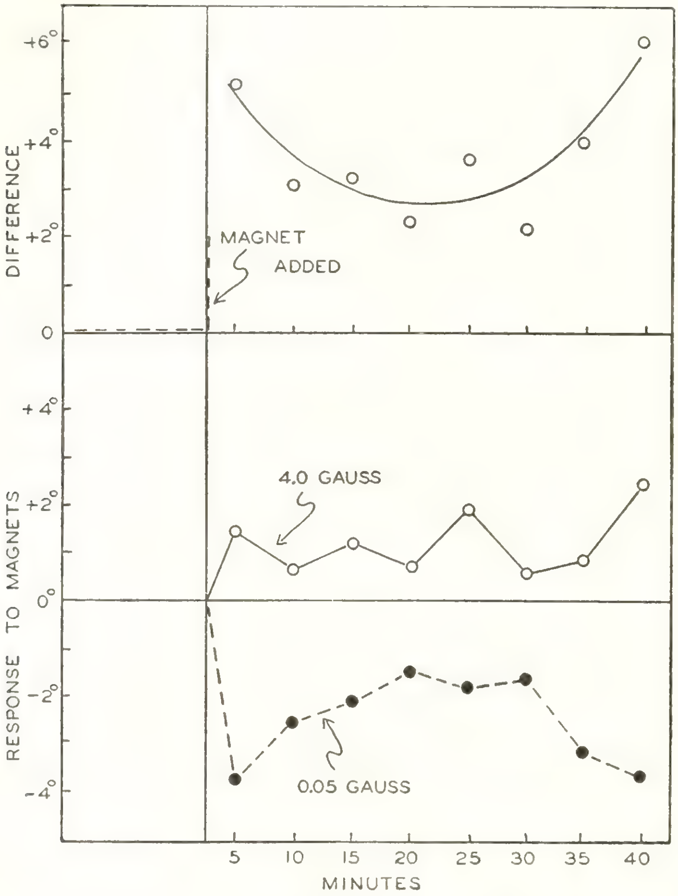

FIGURE 2. A, the response to a reversed 4-gauss horizontal magnetic field, expressed as difference from response to a reversed 0.05-gauss one, during eight consecutive 5-minute intervals during application of the experimental fields. B, the responses to the two strengths of magnetic field, expressed as differences from initial controls, while being subjected to experimental field reversals.

In view of the unexplained difference between the first and second experiment with respect to (a) the amplitude of responses to the two reversed fields and differences between the two responses, and especially (b) the difference between the series relative to the apparent clear response to the 0.05-gauss field in the second, but not in the first experiment, an additional analysis of the data was made. This consisted of computing separately the mean responses to each of the two reversed field strengths while in the field, for each of the 9 months for which data had been obtained. The results are illustrated in Figure 3.

It is apparent from Figure 3 that the responses to the two reversed horizontal-field strengths varied through a relatively large range, even from clockwise to counterclockwise turning. The only common feature was that for every one of the nine months the turning in response to the stronger field was clockwise relative to the response to the weaker one. This was true whether both responses were clockwise, or counterclockwise, or one was clockwise and the other counterclockwise. The apparent absence of a measurable response to the 0.05-gauss field, noted in Figure 1, can now be seen to have resulted from a clockwise turning response during the first three months being balanced by an approximately equal counterclockwise turning response for the last three months. When responses to the two field strengths were in the same direction, the responsiveness tended to vary parallelly, and when in the opposite direction to vary in an opposite or mirror-image manner. Maximum in responsiveness for both field strengths came during Novembers, though for the first November responses to the two strengths possessed the same sign and for the second November they were of opposite sign. In addition to this possible annual variation in strength of the responses, a definite monthly variation in these data was discovered and will be reported later in another connection.

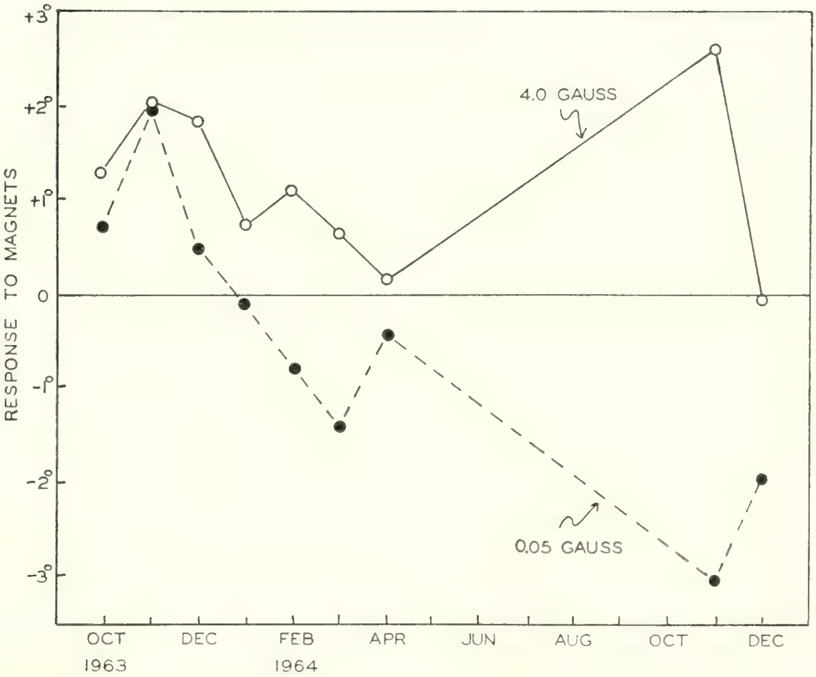

Figure 3. The mean responses to reversed 0.05- and 4.0-gauss horizontal magnetic vectors that were obtained for each of nine calendar months of the investigation in 1963 and 1964.

DISCUSSION

These results confirm in a striking manner earlier reported ones with not only planarians (Brown, 1965) but also mud-snails (Brown, Barnwell and Webb, 1964) in demonstrating a difference between the after-effects on orientational behavior between exposures to reversals of horizontal magnetic vector fields, stronger and weaker than the ambient local one. These field reversals are, of course, reversals relative to all other contemporary ambient vector fields. At the same time, evidence is offered supporting a conclusion that following removal of the experimental field, there is a gradual, exponential decay in the altered state, requiring from 20 to 30 minutes for essentially complete loss.

In addition, this study has suggested strongly that following an abrupt reversal of the horizontal vector of the magnetic field there is an initial maximum response followed by some compensatory reaction which, in the continuing experimental field, proceeds for about 20 minutes. This seems evident in the present experiment principally for the weaker field. This compensatory alteration seems not only to cease but to be followed by a return of the planarians toward a response state of the degree present in the initial response to the abruptly altered field. In brief, there appears to occur a phenomenon which might be termed a "transient accommodation."

Despite the relatively clear demonstration of responsiveness to these weak magnetic fields when essentially simultaneous control and experimental data are compared, and analyses are based upon differences within concurrently obtained data, the drift with time in character of the response to carefully reproduced experimental conditions in the laboratory, such as is illustrated in Figure 3, emphasizes that one is dealing with a biological response within the range of responses normally effected by natural geophysical fluctuations. Other, uncontrolled environmental variables are continuing to exercise influences of the same order of magnitude as the specific ones being investigated, and within these fluctuations are monthly, and suggestively substantial annually, varying components. Variations in these uncontrolled factors are able clearly to modify in a highly significant manner even the response to our controlled experimental alterations. It is of interest to note that November extremes which have been recorded here parallel a late fall extreme value in several other, previously-reported annual variations persisting in so-called constant conditions (Brown, 1964).

One final matter should be mentioned, namely implications of this study for compass responses of animals in unvarying fields of illumination. To the extent that these compass-directional differences in orientational behavior are non-adaptive responses to the earth's geophysical vector fields and depend upon magnetism, this behavioral response would be expected to exhibit variations with time, including not only the already established monthly variations as in snails (Brown, Webb and Barnwell, 1964), but also an annual variation as previously shown for planarians (Brown, 1962, 1963). In addition, longer, secular trends and non-periodic fluctuations of substantial range might reasonably be expected. Similar variability need not be, however, a necessary consequence when adaptive orientational behavior patterns of animals depend upon an informational input from the total contemporary geophysical scene, information which is integrated and interpreted by the adaptively responding organism.

SUMMARY

1. Planarians exhibit a significant difference between response to a reversed horizontal 0.05-gauss and a similarly reversed 4.0-gauss magnetic vector.

2. A difference between orientational behaviors in the natural field following 15-minute exposures of the worms to these two reversed field strengths persists for 20–30 minutes.

3. This after-effect decays exponentially with time.

4. Subjected to an abrupt reversal in horizontal magnetic field, an initial maximal response appears to be followed by a transient accommodation continuing for about 20 minutes. This partial accommodation is lost again completely by the end of 40 minutes. This transient accommodation appeared more evident for the weaker field during these studies.

5. It is shown that the response to the experimental magnetic field reversals varied substantially over the course of the 15-month period of the investigation, in a manner suggesting strongly the existence of relatively large influences of other uncontrolled geophysical variables, factors with biological influences of the same order of magnitude as those being investigated.

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POLYDORA COMMENSALIS ANDREWS—LARVAL DEVELOPMENT AND OBSERVATIONS ON ADULTS[1]

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Although *Polydora commensalis* is often present in the shells of hermit crabs, many aspects of its life-cycle and biology have been overlooked. The larval stages have been incompletely known and there also is confusion as to the mode of fertilization.

The aims of this paper are (1) to describe the larval development of *P. commensalis* so that it might be recognized in waters where it has heretofore been overlooked or confused with other species, and (2) to investigate problems surrounding the annelid's habitat and method of reproduction.

MATERIALS AND METHODS

Since a major aim of this investigation was to describe morphologically the larval development of *Polydora commensalis,* large numbers of the hermit crab, *Eupagurus pollicaris* Say, were obtained. All hermit crabs used in this study were collected in Noank Harbor (U.S.C. & G.S. Chart No. 358).

One hundred and two shells of hermit crabs were opened from November, 1962, to September, 1963, in search of *P. commensalis* egg strings. The four egg strings found provided fertilized eggs and young larvae for laboratory studies.

Stender dishes containing sea water which had been filtered through a Millipore filter, using pads with a porosity of 47 μ (Millipore Filter Corp., Bedford, Massachusetts), were used as rearing vessels. These vessels were immersed in a water bath of running sea water. Sea water in these vessels was changed three times a week. Liver powder was used as a source of food and a freshly prepared suspension was added at the time of each water change (Howie, 1958).

Larvae were also obtained in qualitative plankton tows, using a No. 10 net. Plankton tows were taken on the average of once weekly in front of the laboratory from November, 1962, through December, 1963. Larvae from this source were maintained in the same way as those reared from fertilized eggs.

Descriptions of laboratory-reared larvae and larvae collected from the plankton were made. Larvae were examined in either a hanging drop or under Saran Wrap (Dean and Hatfield, 1963). Larvae of various stages were photographed with a Polaroid camera mounted on a phase microscope. All drawings except

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[2] Appreciation is extended to Dr. David Dean for constructive editorial suggestions and to Capt. L. H. Malloy for providing hermit crabs.

[3] A portion of a thesis submitted in partial fulfillment of the requirements for the M.S. degree from the University of Connecticut, Storrs, Connecticut.

Figure 1 were made from the photographs with the aid of a camera lucida or projector. Figure 1 was drawn from life, using a camera lucida. Larvae were returned unharmed to rearing vessels, following examination.

I. Observations on Adults

a. Distribution

Polydora commensalis was first reported as being present in shells of *Nassarius obsoletus* Say (= *Ilyanassa obsoleta*) inhabited by the hermit crab, *Eupagurus longicarpus* Say, at Beaufort, North Carolina (Andrews, 1891). Andrews also mentions finding this commensal annelid in association with *Eupagurus pollicaris.*

A more recent study reports that this spionid is present along the east coast of Canada, in the shells of *Nassarius obsoletus* occupied by *Eupagurus longicarpus* (Berkeley and Berkeley, 1956).

The author is unaware of any other publications reporting this annelid from the Atlantic coast of North America. However, specimens described in the present paper were found to be very common in various gastropod shells inhabited by *E. pollicaris* at Noank, Connecticut.

The reports of its presence on the west coast of North America are numerous. Berkeley and Berkeley (1936) reported it from Departure Bay, British Columbia, living in shells of *Thais lamellosa* Gmelin inhabited by *Pagurus granosimanus* (Stimpson). Its presence is also recorded in Nanoose Bay in the Nanaimo district, British Columbia (Berkeley, 1927) and from Southern California and Mazatlan, Mexico (Hartman, 1941).

It is further recorded from the North Japan Sea living in shells of *Natica* occupied by a species of *Eupagurus* (Annenkova, 1938). Thus, this annelid is found in several types of shells and in association with more than one species of hermit crab.

In the present study, *P. commensalis* was recorded from the following shells: *Lunatia heros* Say, *Polinices duplicata* Say, *Busycon canaliculatum* Linné and *Buccinum undatum* Linné. In all cases, the crab inhabiting these shells was *E. pollicaris.* Dr. David Dean (unpublished data) also recorded this annelid from Noank Harbor in a *Littorina littorea* Linné shell inhabited by *E. longicarpus.*

b. Habitat

The tubes of *Polydora commensalis* are constructed around the columella of shells by boring a deep furrow and roofing it over with a thin calcareous layer. These tubes are never visible unless the shell is cracked open and frequently extend from the lower part of the columella to the apex of the shell. Fertilized eggs or larvae were found within these tubes, along with an adult worm, during July and August of 1963.

Studies on reproduction, egg deposition, tube-building, etc., would be facilitated if tubes were visible. Therefore, the ability of adult *Polydora commensalis* to build visible tubes on shells and other substrates was tested. All shells used were cleaned by boiling in distilled water. Adult *P. commensalis* were capable of building calcareous tubes on some materials but not on others. All tubes were exposed to view. The results are tabulated below:

Built Tube	Did Not Build Tube
Lunatia heros	*Nautilus pompilius*
Mercenaria mercenaria	Aragonite block
Spisula solidissima	Calcite chips
Thais lapillus	*Crepidula plana*
Littorina littorea	*Crassostrea virginica*
Busycon canaliculatum	*Mytilus edulis*
Polinices duplicata	*Anodonta* sp.

Calcareous tubes were constructed within approximately two weeks. A 3½- to 5-month period was allowed to elapse before a substrate was listed as not conducive to the construction of the calcareous tube of *P. commensalis*.

c. Reproduction

There has been much speculation as to how fertilization is accomplished in *Polydora commensalis*. This is due to reports that there is usually but one individual, always a female, per shell, and that the eggs are found within a concealed calcareous tube (Andrews, 1891; Berkeley and Berkeley, 1936). Individuals approximately 4 mm. in length have been noted by Andrews (1891) and Berkeley and Berkeley (1936). These authors were not able to determine the sex, but suggested that these small worms were males.

The results of this study are in vast disagreement with previous reports concerning number of worms per shell (Andrews, 1891; Berkeley and Berkeley, 1936). *P. commensalis* was found occurring in numbers of two or more per shell 56.7% of the time, and the number reached a total of seven in some instances. Also, these figures are on the conservative side, since it is very probable that some smaller specimens were overlooked. Often two or three of these worms would be found with their tubes side by side.

Furthermore, all *P. commensalis* found were not females. Sometimes only females were present in a shell and at times only males, while on other occasions members of both sexes were present. Sex was determined by presence of eggs or sperm. A total of 27 worms was observed with gametes throughout the winter, spring and summer months of 1963. Ten of these were females and 15 were males. On two occasions both eggs and sperm were found in a single worm. Gravid females ranged in length from 15 to 90 mm. with the average size being approximately 35 mm. Ripe males ranged in length from 3 to 17 mm. with the average length being approximately 12 mm. and the mode 15 mm.

Eggs were light yellow, measured approximately 120 μ in diameter, and were present from segment 14 onward.

All egg masses found within tubes contained fertilized eggs.

II. LARVAL DEVELOPMENT

a. Development in the egg sac

Egg sacs and early development of *Polydora commensalis* Andrews were described by Andrews (1891). However, these stages are presented here, in order to report additional data on ciliation, time of release of larvae to the plankton and other details.

Fertilized eggs and young larval stages are found in the adult tube within partitioned transparent cases (Fig. 1). Approximately 48 hours are required for fertilized eggs to develop into trochophores. At this stage, the beginning of a prototroch is present as two ciliated antero-ventral swellings. A poorly developed telotroch, situated postero-ventrally and laterally, is present as four patches composed of approximately two cilia per patch. The precursor to the vestibule appears anteriorly as a ventral ciliated depression. Neither an apical tuft nor dorsal ciliation are present. Although most trochophores possess two eyes, some specimens are totally lacking in this respect. This variation in number of eyes is evidenced in older larval stages as well. The most outstanding feature of the trochophore is the large yolk mass which comprises approximately 80% of the total body size.

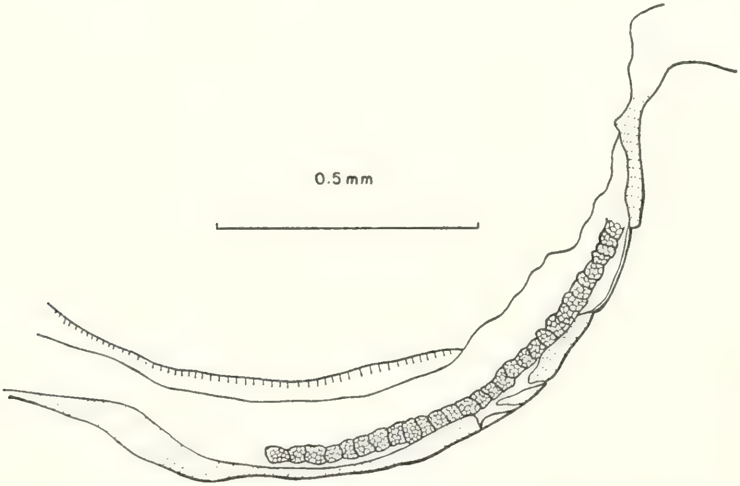

FIGURE 1. Egg string found within adult *P. commensalis* tube. The roof of the calcareous tube was broken in order to expose the eggs. The remnants of the tube are stippled in this figure.

About 24 hours are required for trochophores to advance to 3-segment larvae. At this stage, capillary serrate swimming setae are present on all segments (Fig. 2). A distinct prototroch and telotroch are present, neither of which extends across the dorsal side. The yellow-brown gut is still very yolky, and four eyes are now in evidence. Neither gastrotrochs nor nototrochs have yet appeared.

Two to three days later the larvae have added two achaetigerous segments and become very active (Fig. 3). The capillary notosetae have lengthened considerably and the development of the ciliation makes the larvae very adept swimmers by the 5-segment stage. A short capillary neuroseta appears on setiger 3, marking the transition from a uniramous to a biramous condition. No neurosetae were observed on segments 1 or 2; nor were there either noto- or neurosetae on segments 4 or 5 at this stage. The cilia of the prototroch and telotroch, which extend across the ventral side, have become longer and more powerful. The telotroch is

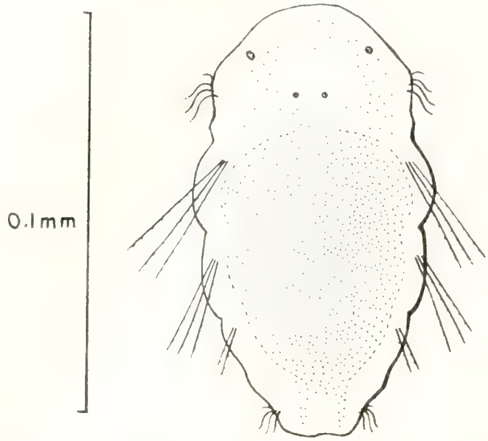

FIGURE 2. Three-segment *P. commensalis* larva. Dorsal view.

composed of distinct patches of cilia. Prominent gastrotrochs, extending across setiger 3 in six patches, and smaller nototrochs make their appearance at this stage. The nototrochs appear to be in six patches across the dorsal side of segments 3 and 4 and in two patches on segment 5. The V-shaped, ciliated vestibule

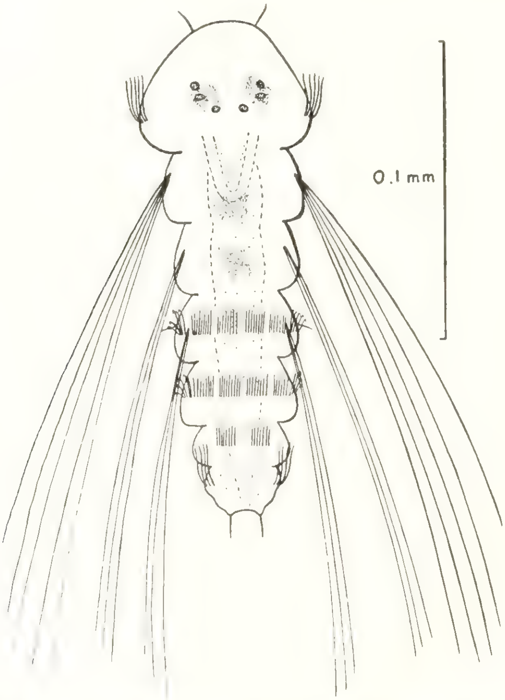

FIGURE 3. Five segment *P. commensalis* larva. Dorsal view.

is now well developed and accentuated by yellow-brown pigment along its border. The larvae ingested liver powder for the first time at this stage. Perhaps the most striking development of the 5-segment larva is the appearance of a median dorsal row of ramified chromatophores. This is characteristic of larvae of all subsequent stages. Almost always these chromatophores are present on every segment, but occasionally they are absent on setigers 1 and 2. The black eyes number four to six and a chromatophore is present between the lateral and median eye on each side. The pygidium assumes a brownish hue and two small anal sensory cilia can be seen projecting from it.

Andrews (1891) suggested that the larvae leave the egg sacs at this stage. It was determined that this was the case by using a method similar to that employed by Wilson (1928) with *Polydora ciliata* larvae. An adult *Polydora commensalis* was left in its calcareous tube with its unbroken egg sacs in a dish of filtered sea water. For the next five days larvae were continually liberated. Twice a day these larvae were collected, examined, and the adult worm put in a dish with fresh sea water. In all cases the collected larvae were at a typical 5-segment stage. Furthermore, the earliest larval stage collected in the plankton possessed five segments, which adds credence to the above results.

b. Development outside the egg sac

The pelagic larvae of *Polydora commensalis* were found in the plankton of the Mystic River Estuary from July to November. Egg strings were found in shells of hermit crabs on four separate occasions during the months of July and August, 1963. The following descriptions characterizing planktonic larvae are based upon examination of many larvae from the above sources. The general description of the pelagic larvae which follows precedes a more detailed description.

One of the most noticeable features of the pelagic larvae is their pigmentation. Planktonic larvae of all stages possess a median dorsal row of black ramified chromatophores on all segments, except occasionally on segments 1 and 2. As the larvae become older and reach the 12- to 16-segment stage, lateral unramified black chromatophores appear on the anterior aspects of each segment. The pigmentation of the vestibule deepens as the larvae develop. The pygidium also appears quite dark. The median row of chromatophores becomes more ramified and spreads across the dorsum in older larvae. The anterior rim of the rounded prostomium is also noticeably pigmented in older larvae. The above pigmentation results in an overall dark appearance of the larvae when viewed with the naked eye or under low power of a dissecting microscope. The only other pigmentation is the black of the eyes. The latter number from zero to six in various specimens, but six is the most prevalent number. On either side there is usually a black ramified chromatophore between the most lateral eye and the eye nearer the midline of the body. One specimen, reared from a fertilized egg to a 28-segment stage, lacked eyes and bore only a median prostomial chromatophore.

The other most obvious characteristic of these larvae is the large size they attain. Specimens surpassing 2 mm. in length are not uncommon.

These general characteristics easily separate the larvae of *P. commensalis* from those of all other spionids reported in the literature to date, except *Polydora hermaphroditica* Hannerz (Hannerz, 1956) and "*Polydora* A" (Gravely, 1909).

The descriptions of these larvae show great similarities to *P. commensalis.* The more detailed examination which follows is necessary, therefore, to avoid confusion.

In early pelagic stages all setae are capillary. Notosetae are much longer than neurosetae. The fifth setiger does not become modified with stout hooks, characacteristic of the genus, until the larva has reached a considerable size. The precise setiger stage at which this modification occurs is variable. These hooks have been observed as minute setae beneath the integument as early as the 15-segment stage. Conversely, they have not been seen in some specimens of 19-segments. However, presence of hooks often is noted between the 18- and 19-segment stage. Associated with the appearance of these modified setae is the reduction in number of capillary neurosetae. The modified setae break through the integument at the 21- and 23-segment stage, and the capillary notosetae are lost.

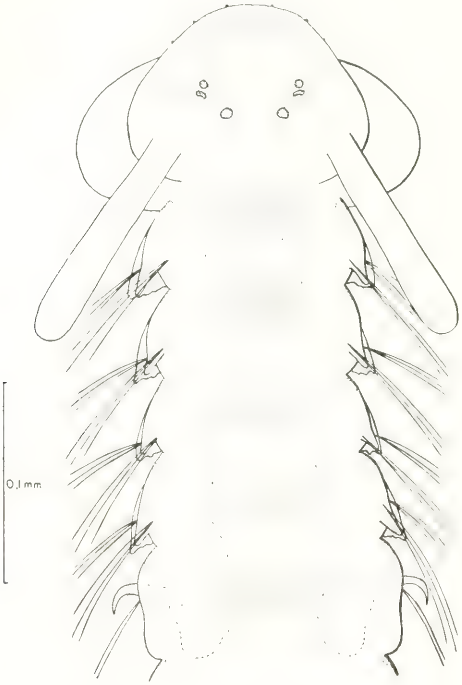

FIGURE 4. Anterior end of a 28-segment *P. commensalis* larva. Dorsal view; ciliation omitted.

Large glands, named "poches glanduleuses" by Claparède (Hannerz, 1956), become noticeable with the advent of the modified setae. These glands enlarge as the hooks increase in size (Fig. 4).

Shortly after the appearance of the modification of the fifth setiger, hooded bidentate crotchets appear in the neuropodia of the posterior segments. These are accompanied by bent capillary setae. On a few occasions the hooded crotchets were observed on larvae with as few as 18 segments. They were always present on larvae of 22 or more setigers and began on the eleventh to fourteenth setigers. All segments have both dorsal and ventral cirri with the exception of the fifth. The ventral cirri are better developed (Fig. 5).

Ciliated swellings lateral to the vestibule become very apparent by the 12-setiger stage. These lateral lips continue to enlarge and in advanced larvae appear enormous when the mouth is wide open.

A prototroch runs across the ventral side of the lateral lips to either edge of the vestibule. It reaches the dorso-lateral edge of the lateral lips, but does not extend across the dorsal side. A minute prototroch is still observed as late as the 28-segment stage. The telotroch extends across the ventral side in patches. All larval stages exhibit a large dorsal gap. Neither a neurotroch nor ciliated pit has been observed at any stage. Prominent gastrotrochs are present in six to eight patches on segments 3, 5, 7, 10, 13, 15, 17, 19, 21, 23, 25, etc. Nototrochs are present in eight patches across the dorsum. The most lateral patch on each side is composed of "grasping cilia" (Wilson, 1928). Usually, nototrochs begin on segment 3 and are present on every segment thereafter. Occasionally, however, a small nototroch is present on segment 2.

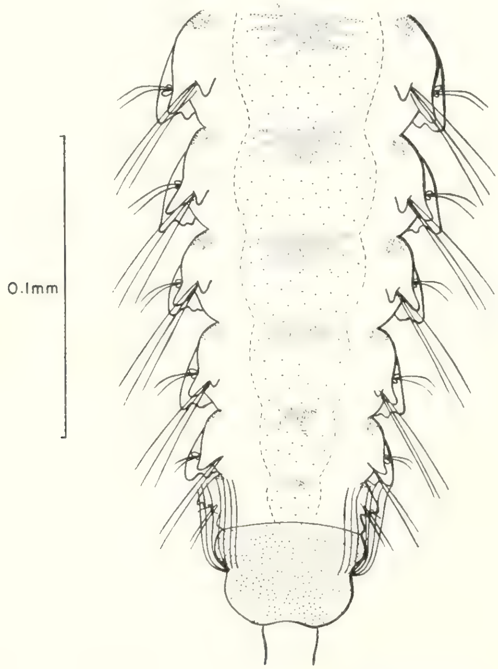

FIGURE 5. Posterior end of a 25-segment *P. commensalis* larva. Dorsal view; nototrochs omitted.

Between the 9- and 10-setiger stage, small swellings, palpi anlage, are observed posterior to the prototroch and anterior to the first segment. These buds gradually increase in length, but remain rather small in relation to the body size, even in the oldest larval stages (Fig. 4). They are greatly reduced compared to other spionid larvae.

Branchial buds are never observed prior to the 22-segment stage. Even at the 28-segment stage they appear quite small, being present on setiger 6 and onward.

c. Metamorphosis and length of larval life

Unfortunately, experiments designed to induce metamorphosis were unsuccessful. Therefore, the length of larval life can be approximated only. Records were kept on the period of time it took for four larvae to develop from fertilized eggs to 27- to 29-segment stages. The results of four separate observations were 42 days, 44 days, 45 days and 38 days. This is an average of 42 days or approximately one and one-half months. After the 27-segment stage is reached, further development is very slow. For example, approximately 15 days were required for a larva to develop from a 27-segment to a 29-segment stage (Table I, specimen 1), whereas observations showed that a specimen might develop from a trochophore to a 14-segment stage in about the same length of time (Table I, specimen 4). The

TABLE I

Estimates of length of larval life of P. commensalis

Specimen no.	Date examined	No. segments	No. days after initial observation
1	July 9, 1963	Fertilized Egg	
	July 28, 1963	14	19
	Aug. 9, 1963	22 to 23	31
	Aug. 13, 1963	24 to 25	35
	Aug. 14, 1963	25	36
	Aug. 20, 1963	27	42
	Sept. 4, 1963	29	57
2	July 9, 1963	Fertilized Egg	
	July 11, 1963	Trochophore	2
	July 12, 1963	3	3
	July 15, 1963	5	6
	July 17, 1963	8	8
	July 29, 1963	14	20
	Aug. 1, 1963	18	23
	Aug. 5, 1963	19	27
	Aug. 10, 1963	23	32
	Aug. 15, 1963	25	37
	Aug. 22, 1963	27	44
3	July 11, 1963	Fertilized Egg	
	Aug. 2, 1963	12	22
	Aug. 25, 1963	28	45
4	July 15, 1963	Trochophore*	
	July 19, 1963	5	4
	July 22, 1963	6 to 7	7
	July 30, 1963	14	15
	Aug. 20, 1963	27	36

* Approximately 48 hours are needed to advance from the fertilized egg to the trochophore stage. Therefore, 2 days should be added to the figures in the column "No. days after initial observation" in order for these figures to correspond to the other 3 observations.

TABLE II

Length-frequency of P. commensalis *adults*

Length (mm.)	Frequency of occurrence	Cumulative
0–1	0	0
1.5–2	5	5
2.5–2	18	23
3.5–4	6	29
4.5–5	9	38
5.5–6	1	39
6.5–7	4	43
7.5–8	8	51
8.5–9	0	51
9.5–10	11	62
10.5–11	0	62
11.5–12	2	64
12.5–13	3	67
13.5–14	3	70
14.5–15	8	78
15.5–16	0	78
16.5–17	1	79
17.5–18	1	80
18.5–19	0	80
19.5–20	4	84
20.5–21	0	84
21.5–22	1	85
22.5–23	0	85
23.5–24	0	85
24.5–25	3	88
25.5–26	0	88
26.5–27	2	90
27.5–28	1	91
28.5–29	0	91
29.5–30	0	91
30.5–31	0	91
31.5–32	0	91
32.5–33	1	92
33.5–34	0	92
34.5–35	0	92
35.5–36	0	92
36.5–37	0	92
37.5–38	0	92
38.5–39	0	92
39.5–40	2	94
40.5–89.5	0	94
90 –90.5	1	95

Notes: The total number of worms collected from shells was 109. The total number of worms where measurements were taken was 95.

latest stage a larva attained in these experiments was 29-segments. After remaining at this stage unmetamorphosed for 21 days, it was preserved.

The length of larval life is probably shorter in nature. By the 22-setiger stage the larvae have acquired all the features of a young benthic worm. The fifth setiger has been modified, hooded crotchets have appeared and branchial anlage are present.

If one assumes larvae are capable of metamorphosing when these features have developed, then the larval life would be reduced to approximately one month (Table I, specimens 1 and 2).

d. Comparison of larvae of Polydora commensalis with Polydora hermaphroditica

The larvae of *Polydora hermaphroditica* (Hannerz, 1956) and "*Polydora* A" (Gravely, 1909) greatly resemble larvae of *Polydora commensalis.* "*Polydora* A" has been reported as identical to *P. hermaphroditica* (Hannerz, 1956).

Larvae of *Polydora hermaphroditica* are very similar to advanced larvae of *Polydora commensalis* in regard to pigmentation, size, ciliation and time of occurrence in the plankton. However, the latter has the following distinguishing characteristics. Branchial anlage are clearly evident from segment 6 onwards. The dorsal median row of ramified chromatophores is present from segment 1 to the posterior end, except on occasion when they are absent on segments 1 and 2. Small unramified chromatophores are present laterally on the anterior side of each segment. They often continue to the posterior end, although at times are lacking in the last few segments. Bidentate hooded crotchets in the neuropods begin between segments 11 and 14, whereas they are found more anterior in *P. hermaphroditica.*

The above characters seem to be the most outstanding features by which to separate the two species. Although the author knows of no reports of the presence of *P. hermaphroditica* in American waters nor *P. commensalis* in European waters, it is important that specimens collected in the plankton be examined critically. It will be interesting to learn whether these species are more widely distributed than reported.

Discussion

Polydora commensalis larvae exhibit typical polydorid characters in regard to morphology, deposition of eggs within the adult tube and planktotrophic pelagic larvae. Inconclusive data indicate that copulation, also typical of the genus, occurs. *Polydora commensalis* has a short period of brood protection followed by a long pelagic life. This species can delay metamorphosis, as shown by one specimen which remained unmetamorphosed at the 29-segment stage for 21 days. Both a long pelagic life and the ability to delay metamorphosis would be advantageous to this species in finding its host.

Adults of *Polydora commensalis* are found in several types of shells and in association with more than one species of hermit crab. This distribution indicates that the commensal relationship is not highly specific. However, one must hesitate in referring to *Polydora commensalis* as a facultative commensal, since there are no reports of adults living in a free state. Usually, when a number of different species may act as host for a commensal, the factors which cement the relationship are quite general in nature (Dales, 1957). In the case presently being discussed, it would appear that the main factors are food and shelter. Furthermore, the host is active and, thus, the more passive annelid obtains benefit by the avoidance of stagnation. The inside of the hermit shell is kept well aerated with fresh sea water. This is accomplished by the current of water from the branchial chamber of the crab, aided by the beating of the pleopods (Jackson, 1913).

The general nature of the relationship was further evidenced by the ability of

P. commensalis to form tubes on various shells which could never be occupied by hermit crabs, *i.e.*, lamellibranch shells. All of the above, plus the fact that this annelid is reported from both sides of the globe, lead to the speculation that *P. commensalis* may be more widely distributed than reported.

During the studies on the reproduction of *P. commensalis* sufficient evidence was collected to speculate that fertilization is accomplished by copulation. In summary, the data tabulated below support this theory.

1. Both males and females are often present in the same shell with their tubes in close proximity.
2. The worms are capable of extending far out of their tubes, thus allowing the sexes to meet and copulation to take place.
3. On occasion, worms in the size range of females are found to contain sperm as well as eggs, indicating copulation had taken place.
4. All egg masses found contained fertilized eggs.

Although the occurrence of several *P. commensalis* of both sexes in one shell seems to have been overlooked previously, the occasional presence of a minute individual approximately 4 mm. in length has been noted (Andrews, 1891; Berkeley and Berkeley, 1936). Andrews had speculated that these minute annelids were males, and hence there was an interesting case of sexual dimorphism in regard to size. However, neither Andrews nor Berkeley and Berkeley were successful in determining if this were the case. In this study, ripe males were found to average a length of 12 mm. On only one occasion were sperm found in worms of 1.5 to 5 mm. in length, though a total of 38 worms were found in this size range (Table II).

SUMMARY

1. *Polydora commensalis* larvae were found in the plankton from July to November, 1963, and fertilized eggs were found in the tubes of adults in July and August.

2. Fertilized eggs develop into 5-segment larvae within 5 to 7 days, at which stage they are liberated to the plankton.

3. Ciliation, setation, pigmentation and other taxonomically important features are described for larvae from the trochophore to 29-segment stage. Planktonic larvae of all stages possess a dorsal median row of black ramified chromatophores. Advanced larvae may exceed 2 mm. in length. The pigmentation and large size attained are the most outstanding characteristics of *P. commensalis* larvae.

4. Due to the similarity between larvae of *P. commensalis* and *P. hermaphroditica,* a comparison of the two is given.

5. The length of larval life of *P. commensalis* is estimated to be between one and one and a half months.

6. Data on distribution, habitat and reproduction are reported. It is suggested that this annelid is more widely distributed than reported, and that fertilization is accomplished by copulation.

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THE CYTOLOGICAL EFFECTS OF PODOPHYLLIN AND PODOPHYLLOTOXIN ON THE FERTILIZED EGGS OF CHAETOPTERUS[1]

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One of the clues to the mechanism of mitosis is a study of the ways in which it can be blocked; this has been discussed by Wilson (1963). There have been many such studies, utilizing a wide variety of chemical and physical agents; one of the best known of these agents is colchicine, which has been extensively employed by Eigsti and Dustin and their co-workers, and by many others. Colchicine apparently acts by blocking the mitotic division at metaphase, destroying the spindle in the process; chromosome replication is not affected and polyploidy may result. Podophyllin (derived from the mandrake root) is a similar mitotic poison which destroys the spindle (Sullivan and Wechsler, 1947); however, polyploidy has apparently not been reported to be a sequence of its use. Cornman has made extensive investigations into the action of this substance (reviewed by Cornman and Cornman, 1951) and of related or derived compounds, such as podophyllotoxin and quercetin. The early interest in these substances derived from their apparent efficacy against certain forms of warts *(condylomata acuminata)* and, later, against cancerous cells (see Cornman and Cornman, 1951, and Biesele, 1958, 1962, for specific references). The latter application has not proved to be especially valuable, but podophyllin and its derivatives are nevertheless of considerable interest as antimitotic agents, because of their great potency at very low concentrations.

Biesele (1958) has extensively reviewed the literature on the action of mitotic poisons, including podophyllin and its derivatives, and colchicine. He lists the following agents as among the poisoners of metaphase and later stages of mitosis: colchicine and its derivatives, a number of physical agents, many organic compounds, podophyllin and related compounds, certain sulfhydryl reagents, quinones and phenols, antifolic acids, etc. Of these, we elected to test podophyllin and podophyllotoxin (the active principle of crude podophyllin resin), using fertilized eggs of the polychaete annelid, *Chaetopterus.* With this biological material, it was possible to have a large population of relatively uniform cells, all of which were at the metaphase of the first meiotic division (Figs. 1 and 2) and therefore at a susceptible stage for an antimitotic agent of this type. Cornman and Cornman (1951) used the eggs of certain echinoids (*Arbacia, Lytechinus, Tripneustes* and *Echinarachnius*), of the asteroid, *Asterias,* and of the gastropod, *Chromodoris.* In none of these experiments, with the exception of some tests with *Asterias,* were the eggs at the first meiotic metaphase after fertilization when they were treated.

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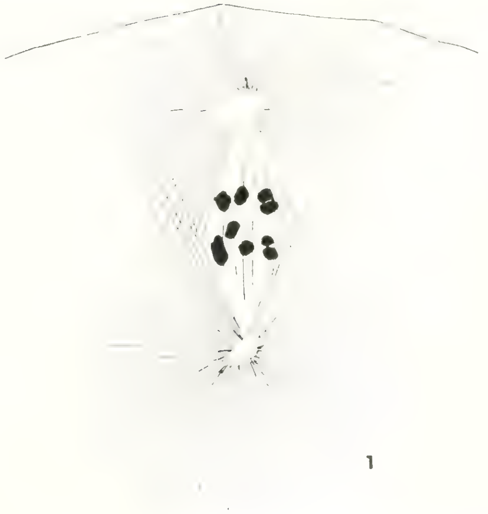

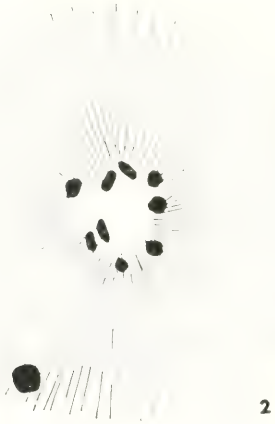

PLATE I

All drawings in this and the succeeding plates are of fixed *Chaetopterus* eggs (whole-mounts, slightly compressed), drawn with the aid of a camera lucida, using 20 × oculars and an oil immersion or a 4-mm. objective, giving total magnifications of 3200 or 1300, respectively. The eggs illustrated were fixed in Kahle's fluid and stained lightly in Harris' acid haematoxylin.

FIGURE 1. Normal first maturation metaphase in *Chaetopterus,* seen in side view. The nine maturation chromosomes are easily countable. Magnification: 1600 ×.

FIGURE 2. Polar view of a normal first maturation metaphase. The aster at only one of the spindle poles is shown. The large black body at the lower left is the nucleolus (which is not shown in Figure 1). Magnification: 1600 ×.

We are indebted to Miss Marion Seiler for her assistance in preparing the final drawings for publication, and to Mr. Donald E. Kent for photography and for his help in other phases of the investigation.

METHODS

Eggs and sperm of *Chaetopterus pergamentaceus* were obtained and inseminated in freshly filtered sea water by the methods outlined by Costello *et al.* (1957). Exactly five minutes after insemination (at which time the egg is at metaphase, preparing to undergo the first meiotic division) measured amounts of podophyllin [2] or podophyllotoxin [2] solution were added to 200 ml. of egg suspension, using a volumetric pipette, while the dish contents were being thoroughly mixed. Both control and experimental dishes were kept on the sea water table; air temperatures varied from approximately 20° C. to 25° C. for different experiments but were relatively constant during the course of any one experiment. At the conclusion of the desired period of treatment, the eggs were washed with large quantities of freshly filtered sea water, and allowed to continue development. Control eggs were cultured in freshly filtered sea water and, except for the absence of the antimitotic agents, were handled in a manner similar to that used for the experimental ova. Treatments were, in most cases, one hour in duration, except where otherwise noted.

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[2] We wish to express our appreciation to Mr. George Motoasca, of S. B. Penick & Company, for samples of these drugs used in the earlier portion of our studies.

Development of the living control and experimental eggs was observed for periods up to three hours, and at intervals after that. Egg-samples were fixed at various times, calculated so that the ova would be killed at the metaphases of first and second polar body formation, and of first and second cleavages. Samples were also fixed approximately 24 hours after insemination, to ascertain what degree of later development, if any, had occurred. Kahle's fixative was routinely used, and the eggs were killed directly on #1 coverslips, by the method described by Henley and Costello (1957). The preparations were stained with Harris' acid haematoxylin, dehydrated in an ethanol series and mounted in damar or Permount. Camera lucida drawings (Figs. 1–17) were made of living and fixed eggs as records; approximately 2100 permanent preparations were studied, utilizing an oil immersion or a 4 mm. objective and 20 × oculars.

The podophyllin and podophyllotoxin stock solutions were made up as follows. Ten mg. of the dry drug were diluted in 10 ml. of distilled water, in a volumetric flask. Appropriate amounts of these stock solutions were then further diluted in volumetric flasks with distilled water to the desired concentrations (0.1 to 0.00001 mg./ml., final concentration, when added to 200 ml. freshly filtered sea water-egg suspension). All solutions were kept refrigerated until shortly before use, when they were allowed to come to room temperature.

Fifty-five series of experiments were carried out.

RESULTS

The sequence of early events in the cytological effects of podophyllin

In fertilized *Chaetopterus* eggs treated with high to moderate levels of podophyllin (0.1 to 0.005 mg./ml.), a characteristic series of early events takes place (Figs. 3–11). In eggs fixed as soon as one minute after the beginning of treatment (with a dosage of 0.005 mg./ml.), there is a suggestion of fading of the asters of the egg maturation figure; the chromosomes look normal in such eggs. At 1½–2 minutes after the beginning of treatment, this process of fading continues (Figs. 3, 9), so that by two minutes after the initiation of treatment, some of the maturation figures appear almost normal, while in others one or both asters are faint or absent, or slightly smaller than normal (Fig. 8). At higher dosages (0.01 mg./ml.), the spindles and asters have disappeared by three minutes after the beginning of treatment (Fig. 5), although the chromosomes remain in essentially the normal first maturation metaphase configuration. By 5 minutes after the beginning of treatment, what appears to be a faint "membrane" begins to appear around the group of chromosomes (Fig. 6), which have now begun to take on a somewhat vesicular appearance, but which still usually remain oriented in a ring of 9 around the periphery of the former spindle area. This same process of "membrane" formation begins about a minute later in eggs treated with lower doses of podophyllin. The containment of the chromosomes within a common membrane (Fig. 7) remains more or less unchanged for a period of a little less than half an hour, after which the chromosome vesicles are released, to lie loose in the cytoplasm (Fig. 10). Cornman and Cornman (1951) described a somewhat similar series of events in

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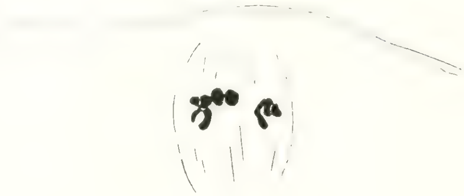

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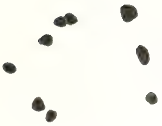

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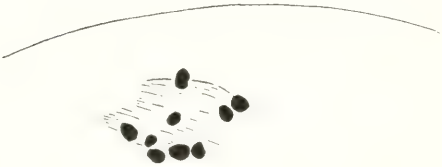

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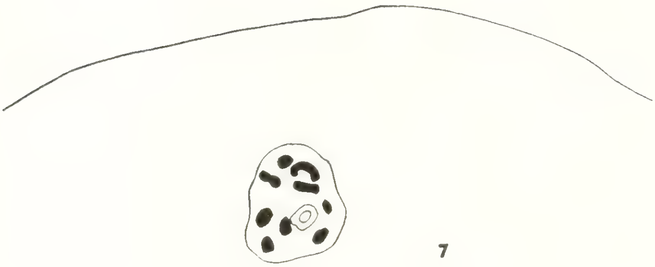

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PLATE II

Illustrations in Plate II are of ova from the same experiment, treated with 0.01 mg./ml. podophyllin, beginning five minutes after insemination. Magnification: 1600 ×.

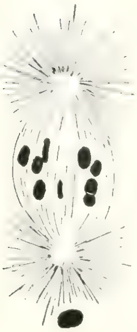
FIGURE 3. One aster (the inner one) has completely disappeared, and part of the first maturation spindle (seen in side view) is likewise no longer visible, in this egg fixed 90 seconds after the initiation of treatment. The large round black body is the nucleolus. Only 8 of the haploid complement of 9 chromosomes could be definitely counted.

FIGURE 4. In this egg, fixed three minutes after the beginning of treatment, both asters have entirely disappeared, and the maturation spindle (side view) is only faintly visible. The fertilization membrane is present at the surface of the egg. (The wrinklings of this membrane, described in the text as typical results of podophyllin treatment, are not evident in this fixed material.) It appeared that only 8 of the 9 chromosomes were present, but it is likely that one of the larger masses of chromatin actually represents two chromosomes.

FIGURE 5. All 9 chromosomes are easily countable, in this polar view of the characteristic first maturation metaphase ring configuration. Both asters and the spindle have completely disappeared, and in the preparation there is only a slight staining at the center of the ring to indicate where the spindle substance was formerly located (not indicated in the drawing). Sample fixed three minutes after the beginning of treatment.

FIGURE 6. In this side view of a first maturation metaphase, the 9 maturation chromosomes are countable, and there is a suggestion (at top) of "membrane" formation. Sample fixed 5 minutes after the beginning of treatment.

FIGURE 7. "Membrane" formation is now completed and the 9 egg maturation chromosomes are entirely enclosed therein, as is the nucleolus (the lighter ring structure). Sample fixed 31 minutes after the beginning of treatment; at this stage, the control eggs were at the metaphase of the first cleavage. Note the similarity in diameter of the chromosome group in this treated egg to that of the normal first maturation metaphase (Fig. 2).



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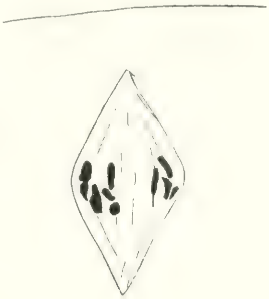

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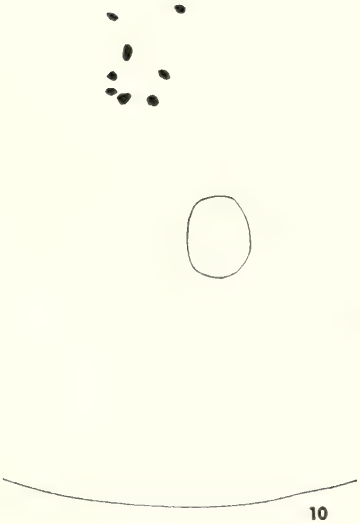

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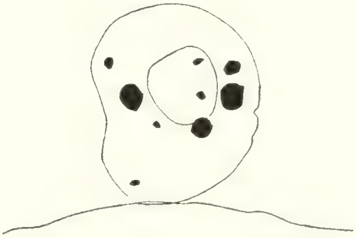

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Plate III

The eggs shown in this plate are from four different experiments, and were treated with varying concentrations of podophyllin (noted for each case); all treatments were begun five minutes after insemination. Magnification: 1600 ×.

Figure 8. There is marked reduction in the diameter of the asters and in the length (but, apparently, not of the diameter) of the maturation spindle, in this side view in an egg from a sample fixed three minutes after the beginning of treatment with 0.0055 mg./ml. podophyllin. The nucleolus is visible near the lower aster.

Figure 9. Side view of a first maturation metaphase. The spindle is still clearly demarcated but the asters have entirely disappeared. Sample fixed three minutes after the initiation of treatment with 0.01 mg./ml. podophyllin (from the same experiment as the eggs shown in Plate II).

Figure 10. The egg maturation chromosomes are now lying free in the egg cytoplasm, with no vestige of asters or spindle. The remnant of the "membrane" which formerly enclosed them (see Figure 7) is lying loose in the cytoplasm nearby. Only 8 of the 9 chromosomes could be counted with certainty. Sample fixed 21 minutes after the beginning of treatment with 0.1 mg./ml. podophyllin.

Figure 11. In this egg from a sample fixed 115 minutes after the initiation of treatment with 0.0055 mg./ml. podophyllin, the egg chromosomes have become segregated in a large exovate, simulating a polar body (although very much larger than a normal polar body). There has been some fragmentation and, perhaps, re-consolidation of the chromatin, judging from the variation in size of the chromatin bodies.

living *Asterias* eggs treated with podophyllin at the first maturation division. This process of release occurs first for the egg chromosomes, but somewhat later (approximately an hour after the beginning of treatment with 0.005 mg./ml.) it is also evident in the sperm chromosomes which have also become surrounded by a membrane. (See, also, Cornman and Cornman, 1951.) The two groups of chromosome vesicles often eventually come to be intermingled and indistinguishable from one another. The exact sequence of events here appears to depend upon the entrance point of the sperm, with reference to the maturation figure, inasmuch as the two groups of chromosomes sometimes remain discrete. The empty "membranes" remain visible for a brief period (Fig. 10) but eventually disappear.

Occasionally, there is some fusion of the egg chromosome vesicles with one another, so that fewer but larger vesicles are visible (Fig. 4). Usually, however, nine (the haploid number for *Chaetopterus*) can easily be counted. Cornman (1949) has also reported this apparent fusion of chromosome vesicles in living *Asterias* eggs which had been treated with podophyllin.

The disappearance of the asters and spindles is so complete that there remains no evidence of these structures, except for a slightly more homogeneous staining of the general area they formerly occupied. (By the methods used, there is almost always some staining of the egg cytoplasm with haematoxylin, although this is not sufficiently great to interfere with observations.)

The general sequence of events, as described above, occurs after almost all except the low doses of podophyllin (0.00005–0.00001 mg./ml.), although the tempo is noticeably faster in eggs treated with the higher concentrations. There is no further visible change in the eggs, and no further development occurs at doses between 0.01 mg./ml. and 0.0025 mg./ml. There was no evidence of any conspicuous cyclic growth, disappearance and reappearance of chromosome vesicles, such as were described by Cornman and Cornman (1951) for podophyllin-treated echinoderm eggs. There was no recovery of treated eggs in our experiments, in contrast to the results reported by Inoué (1952) for colchicine-treated *Chaetopterus* ova.

The most striking feature of these results is the remarkable rapidity with which effects are evident, as soon as 60 seconds after the initiation of treatment. This must mean that podophyllin penetrates the *Chaetopterus* egg very rapidly indeed. Swann and Mitchison (1953) found a comparable rapidity of effect of colchicine in causing the achromatic figure to disappear in treated *Psammechinus* eggs, as did Inoué (1952) for colchicine-treated *Chaetopterus* eggs.

The source of the "membrane" which comes to surround the chromosomes is an interesting problem; we are inclined to suspect that it may be derived from the spindle substance, but there is no real evidence for or against this theory except for the suggestion afforded by Figure 6. Gaulden and Carlson (1951) described the formation of a hyaline globule from the spindle substance of colchicine-treated grasshopper neuroblasts, and Kobayashi (1962) reported the complete disappearance of the spindle in demecolcine-treated *Mespilia* eggs, leaving a hyaline zone, which stained poorly with haematoxylin, around the nucleus. In Kobayashi's experiments, there was recovery from the effects of the mitotic poison, and during the early phases of this recovery process, the hyaline material was associated with the reconstituted spindle, spread over the fibrous structures of the mitotic apparatus.

At each of the first three or four divisions after recovery, the hyaline material was distributed to each of the daughter cells, after which it disappeared.

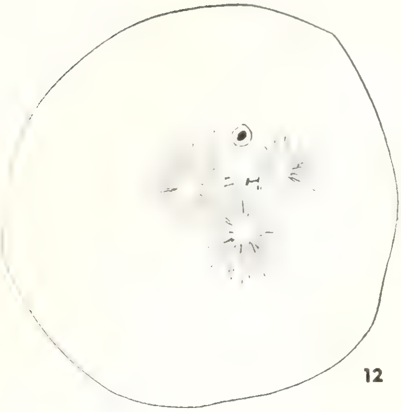
It is interesting that although there is a pronounced reduction in the length of the maturation spindle in podophyllin-treated eggs (compare Figures 1 and 8, for example), the diameter is apparently not affected (compare Figures 2 and 5). (See, also, Inoué, 1952.) Furthermore, the chromosomes usually retain the normal maturation metaphase configuration. This affords confirmation for the idea that the spindle fibers are oriented longitudinally (Inoué, 1952, 1953).

As shown in Figures 3 and 4, the asters were almost invariably the first structures affected by podophyllin, the spindle persisting for as much as one and one-half to two minutes longer. It was observed, also, that the first aster to be affected was usually (but not always—see Figure 3) the one which was nearest the periphery of the egg. It seems reasonable to believe that this is because the antimitotic agent would penetrate through the egg surface and first encounter the more peripheral of the two maturation asters. A similar susceptibility of *Arbacia* egg asters to the effects of another antimitotic agent, colchicine, was reported by Nebel and Ruttle (1938) and by Beams and Evans (1940). Sauaia and Mazia (1961) have demonstrated that in isolated sea urchin egg spindles, previously treated with colcemide (which is very close in chemical structure to colchicine), the asters are likewise the first components of the achromatic figure to be affected. Colcemide applied to the spindles after isolation resulted in no changes, which is not surprising. Beams and Evans (1940) found that the spindle was also destroyed by colchicine, but somewhat later than the asters. They attribute the destruction of asters and spindles to a solation of the cytoplasm, which causes destruction of the spindle, leaving the chromosomes relatively unaffected.

In addition to the rapidity of the effects of podophyllin, another very striking feature is the completeness of the effects, the chromosomes and nucleoli alone remaining more or less unaffected (except for the somewhat vesicular appearance the chromosomes assume). There is no remaining trace of the asters, not even as the "lakes" described by Beams and Evans (1940) as resulting from colchicine-treatment of *Arbacia* eggs. The only evidence of the spindle which persists, as noted above, is a somewhat more homogeneous staining of the egg in the region formerly occupied by this structure. We found no evidence of any fibrous structure in this area; however, its boundary, which we have described as resembling a membrane, is very sharp. It seems quite likely that the association of the chromosomes and the spindle fibers or their remnants may remain in effect somewhat longer than would be apparent at first glance, since the chromosomes retain their characteristic ring-shaped metaphase arrangement for some time after the spindle has visibly disappeared (Fig. 5).

The effects of low doses of podophyllin (0.00005 mg./ml.–0.00001 mg./ml.)

After treatments with concentrations of 0.00005 mg./ml., for periods of 40 to 50 minutes, there was some recovery in treated eggs, although even with these very low doses, the resulting trochophores were usually atypical, with marked ciliary defects and other abnormalities. There was some evidence, also, of fusion of several embryos to form a single "giant." By 24 minutes after the beginning of treatment, there was reduction in the size of the first cleavage asters in some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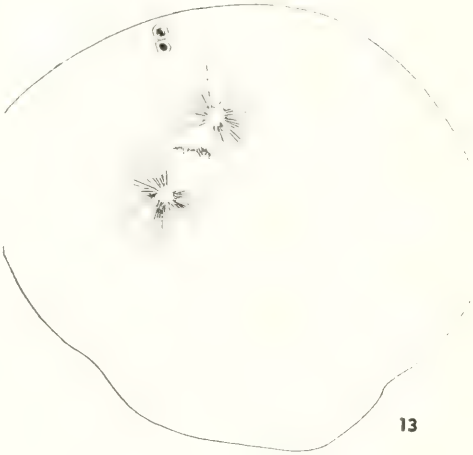
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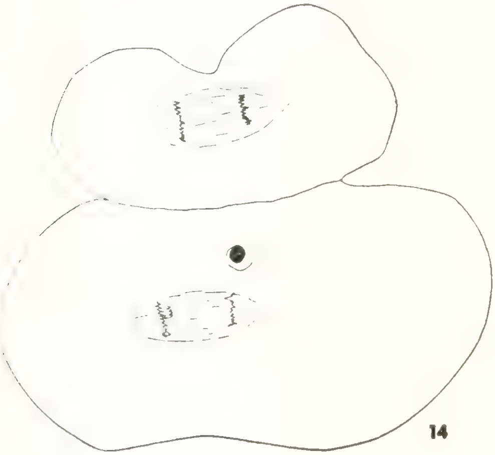
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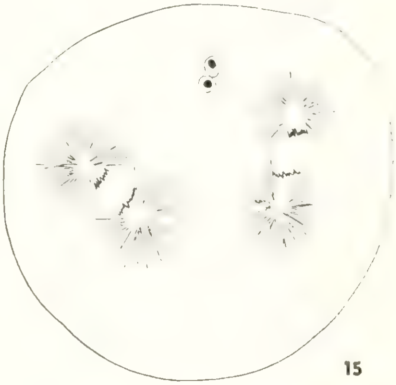
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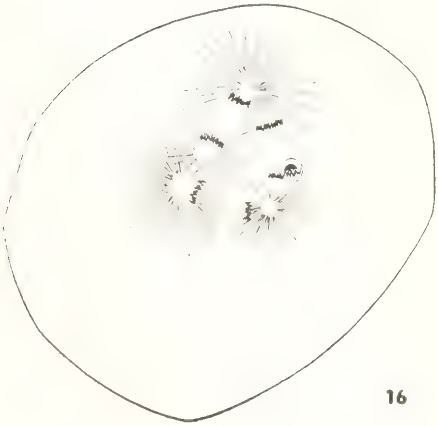
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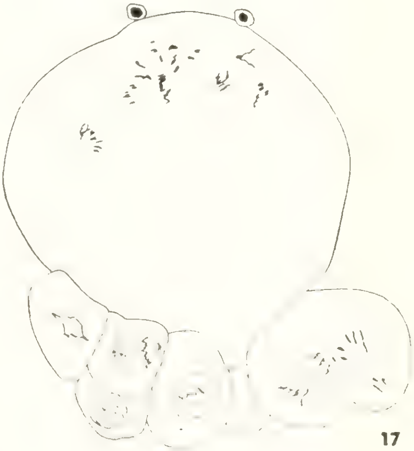
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PLATE IV

eggs, while in others, these structures remained essentially normal. At 47–57 minutes there were some multipolar cleavage figures, and in other eggs, two separate complex metaphases (perhaps male and female in origin?) were apparent. Again, the asters and spindles were fairly normal in a number of ova, but reduced in size in others (see, also, Gaulden and Carlson, 1951, and Kobayashi, 1962). Sixty-eight minutes after the beginning of treatment, much variation was apparent: some eggs had a single large complex metaphase, some had several multipolar figures, some had from one to six interphase nuclei. Usually it appeared that no cytokinesis whatever had taken place, or that it had been partially suppressed, at least. By 113 minutes after the beginning of treatment, the cytological picture was much like that observed at 68 minutes, except that more interphase nuclei (up to 8 in one egg) were observed. Occasionally, cytokinesis had taken place—often resulting in an unbalanced distribution of chromatin, so that one of the daughter "cells" had none.

Treatment with a still lower concentration of podophyllin (0.00001 mg./ml.) (Figs. 12–17) resulted in the appearance of a number of multipolar cleavage figures (Fig. 12) at 27 minutes after the beginning of treatment, and in occasional reduction in size of the asters and spindles (Fig. 13); otherwise, the eggs appeared essentially normal. At 52 minutes after the beginning of treatment, some relatively normal two- (Fig. 14) and four-cell stages, with both polar bodies, were found; other eggs had multiple or multipolar figures in non-divided cytoplasm (Figs. 15,

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All the eggs shown in this plate are from the same experiment, treated for 60 minutes with a low concentration of podophyllin (0.00001 mg./ml.), beginning five minutes after insemination. Magnification: 650 ×.

FIGURE 12. Tripolar figure, from an egg-sample fixed 27 minutes after the beginning of treatment. The control eggs fixed at this time were in the prophase to metaphase of the first cleavage, so it seems likely that the mitotic figure shown here is likewise a cleavage spindle, since the development of experimental and control eggs in this series was quite closely synchronous at this point. The chromatin is scanty and thread-like. One polar body is visible at the top of the figure, overlying it on the surface of the egg.

FIGURE 13. In this egg, fixed at the same time as the one shown in Figure 12, the asters are smaller in diameter than usual, but otherwise the figure is similar to the cleavage metaphases in the control eggs. Both polar bodies are present.

FIGURE 14. Two-cell stage, at the anaphase of the second cleavage. The asters are not visible at the ends of the spindle; this second cleavage was synchronous with the same division in the control eggs. One polar body is visible on the larger CD blastomere, below. Fixed 52 minutes after the initiation of treatment.

FIGURE 15. There are two entirely separate anaphase figures in this uncleaved egg fixed at the same time as that shown in Figure 14. No cytokinesis has taken place, but karyokinesis is proceeding, although the two discrete figures are somewhat smaller than normal (compare with Figure 14). Both polar bodies are present (at the top).

FIGURE 16. This complex anaphase configuration is from the same egg-sample as those ova shown in Figures 14 and 15 (fixed 52 minutes after the beginning of treatment). Control samples fixed at this time were proceeding from the two- to the four-cell stage. At least three spindles can be discerned, their relationship to one another being somewhat obscure. One polar body is present beneath the complex array of spindles, at the right.

FIGURE 17. In this egg, fixed 115 minutes after the beginning of treatment (when the controls were in advanced cleavage stages with ±16 cells), abnormal cytokineses has occurred, with six smaller cells in association with one larger one. Two interphase nuclei can be seen in two of the small cells, and most of the mitotic figures appear to be abnormal, including one or more multipolar spindles in the larger cytoplasmic mass. Both polar bodies are present on the periphery of the egg. The larger of the associated cells is undergoing an atypical anaphase.

16). By 115 minutes after the initiation of treatment, there were occasional normal advanced cleavage stages, and other ova with partially or wholly undivided cytoplasm. Quite often, suppression of cytokinesis had occurred only to a degree, although karyokinesis in such cases was usually highly abnormal (Fig. 17). A few multipolar cleavage figures were also observed. The further development of all these eggs was variable; some fairly normal trochophores resulted, while others had marked ciliary defects and there were many dead larvae.

It is important to emphasize here the fact that although there was considerable recovery in the eggs treated with these very low doses, subsequent development was not entirely normal (Figs. 12–17) and the resulting trochophores were almost invariably atypical to at least some degree. Thus, there must be quite profound effects on the egg which are not necessarily apparent at its early stages of development, except in the reduction in size of the spindles and asters.

The multipolar figures observed in cleavage stages of the treated eggs are very similar to those which occur in *Chaetopterus* ova after a number of other experimental treatments, including x-rays (Henley and Costello, 1957) and low temperature (Henley, 1959). They have also been described in the eggs of other forms after a wide variety of experimental treatments, and in rat ascites tumor cells treated with podophyllin (Makino and Tanaka, 1953). Similarly, the suppression of cytokinesis, but not of karyokinesis, also occurs after such treatments.

The fact that the polar bodies usually appeared in a more or less normal fashion (temporally and spatially) after low doses of podophyllin suggests that since the achromatic figures were not usually affected as early after the beginning of treatment with podophyllin at low concentrations as at higher concentrations, polar body formation could proceed in an essentially normal manner. It is of interest that even in eggs treated with higher concentrations of podophyllin (see following section) there was a simulation of polar body formation (Fig. 11); such pseudopolar bodies were often resorbed, however.

Shape changes in podophyllin-treated eggs; effects on the egg membranes

After treatment with even moderate to high doses of podophyllin, *Chaetopterus* eggs exhibited remarkable simulation of the normal shape changes which characterize the development of this form, even though maturation and cleavage spindles and asters were completely inhibited. With medium concentrations of podophyllin (0.0055 mg./ml., for example), a semblance of polar body formation took place, in which the entire egg nuclear complex was segregated into a large exovate (considerably larger than a normal polar body) (Fig. 11); this exovate usually remained attached to the egg cytoplasm by a stalk. Such "pseudo-polar body formation" occurred at the same time after insemination as the formation of a normal first polar body in the controls. The pseudo-polar body was often resorbed into the egg cytoplasm within about 10 minutes after its initial appearance, and there was usually (but not always) no repetition of the event at the time of second polar body formation in the controls. In occasional ova, exovates occurred in which no chromatin was present, as ascertained by examination of cytological preparations fixed at the time of exovate formation. It is possible that such exovates represent a rupture where the egg cortex and membrane were interrupted at the point of ovarian attachment. Inoué (1952) has described similar pseudo-polar body formation in colchicine-treated *Chaetopterus* eggs.

Concurrent in time with the characteristic "pear" and polar lobe stages of the control eggs, there were very normal-appearing similar stages in the experimental eggs treated with moderate dosages of podophyllin. The polar lobes were subsequently resorbed by the eggs, approximately 20 minutes after they had appeared, and no further development occurred. Cytological examination of such treated eggs showed that in many cases, at least, the shape changes could be correlated in time with the release of the chromosome vesicles from the membrane. In some instances, abortive cleavages were initiated in the experimental eggs, but except after low doses, such furrows were resorbed within 10–15 minutes.

Similar shape changes in podophyllotoxin-treated eggs, simulating those of normal control eggs, were also observed.

Particularly after treatment with the higher doses of podophyllin (0.1–0.005 mg./ml.) striking effects on the eggs' membranes were observed. Following a dose of 0.1 mg./ml., for example, the membranes wrinkled around the entire peripheries of the eggs, at 57 minutes after the beginning of treatment. Seven minutes later, this wrinkling became very pronounced, and by 157 minutes after the beginning of treatment with this dosage, the membranes became symmetrically exaggerated in their elevation from the surfaces of the eggs. Subsequently, the symmetrical exaggeration often became asymmetrical. The general course of events in this process of exaggerated membrane elevation was strikingly reminiscent of that observed in *Chaetopterus* eggs after cold treatment (Henley, 1959), except that the ova usually did not become denuded of their membranes after podophyllin treatment, as was often the case after cold treatment.

There is a normal series of wrinklings in the *Chaetopterus* egg membrane after fertilization (Pasteels, 1950), but the exaggerated crenations observed after podophyllin treatment are very much more conspicuous.

There is no demonstrable relation between the integrity of the mitotic apparatus and the occurrence of shape changes in the podophyllin-treated *Chaetopterus* egg. In the normal egg of this annelid, the pear-shaped stage coincides with early metaphase of the first cleavage division and the polar lobe stage lasts from approximately the metaphase to anaphase of the same division. Similarly, the appearance of pseudo-polar bodies without chromatin is apparently entirely divorced from the presence of the functional maturation spindle and chromosomes in the egg; this is shown by the fact that in eggs fixed at the time when pseudo-polar bodies were present, the asters and spindles had disappeared (probably about 8–10 minutes earlier judging from the evidence obtained concerning the rapidity of effect of higher concentrations of podophyllin). The ultimate resorption of pseudo-polar bodies and of polar lobes may mean that although the initiation of these morphological changes can occur in the absence of the spindle and asters (but, it should be noted, in the continuing presence of the chromosomes), it cannot continue to the point where polar bodies are actually given off as a consequence of cytokinesis.

The observed exaggerated membrane elevation may result from a separate effect of podophyllin on the cell surface, as distinguished from the effects on asters and spindles.

An attempt to reverse the antimitotic action of podophyllin

The possible antagonistic action of reduced and/or oxidized glutathione was studied, in an attempt to reverse the antimitotic action of podophyllin (see Kawamura, 1960, and Zimmerman, 1960). *Chaetopterus* eggs were subjected to treatment with concentrations of podophyllin (0.012–0.00032 mg./ml.) known to be effective in destroying astral rays; they were then treated, two to five minutes later, with various concentrations of oxidized (0.25–0.025 mg./ml.) or reduced (0.25–0.05 mg./ml.) glutathione. In another series of experiments, the oxidized or reduced glutathione was added to the eggs concurrently with the podophyllin. In no case (regardless of concentration) was there a clear-cut reversal or prevention of injury to the amphiastral system. In some cases (high dosage—0.25 mg./ml.) the effects of oxidized or reduced glutathione plus podophyllin in inhibiting cleavage were greater than those after treatment with podophyllin alone. Such high doses of glutathione, administered to eggs in the absence of podophyllin, were injurious, also.

There is thus no indication that glutathione, either in reduced or oxidized state, can reverse the antimitotic effects of podophyllin, under the conditions of these experiments.

The effects of podophyllotoxin

In general, the effects of podophyllotoxin are entirely comparable to those observed after podophyllin treatment, although the requisite concentrations of podophyllotoxin for a given effect are lower for the active principle than for the crude resin (Table I). A range of concentrations, from 0.01 mg./ml. to 0.00005 mg./ml., inclusive, was tested. In a typical experiment, utilizing podophyllotoxin at a concentration of 0.001 mg./ml., for a duration of approximately 83 minutes, the general cytological picture was like that seen in eggs treated with much higher dosages of podophyllin (0.01 mg./ml., for example); the podophyllotoxin-treated ova showed discrete chromosome vesicles within a membrane, occasional appearance of a large pseudo-polar body, and eventual release of the chromosome vesicles, so that they were lying loose in the egg cytoplasm, by 55 minutes after the beginning of treatment. With a lower concentration of podophyllotoxin (0.00025 mg./ml.), a normal first maturation metaphase was observed in treated eggs as late as 7 minutes after the initiation of treatment, and a normal-appearing second maturation metaphase also appeared at the usual time. By 40 minutes after the beginning of treatment, the first cleavage spindles had disappeared; here it was observed that the spindles were reduced in length and perhaps in diameter. By 65 minutes after the beginning of treatment, only three of 30–40 eggs on a given slide had cleaved and were at a normal-appearing two-cell stage. In the others, no spindles, asters or chromosomes could be seen. Polar bodies were sometimes present on the treated ova, sometimes absent. By 115 minutes after the beginning of treatment, at least one cleavage had occurred in nearly every egg, with most developing ova being at 4-, 6- or 8-cell stages. There was a pronounced delay in cleavage of the treated eggs, as compared with the controls.

After a dose of 0.0005 mg./ml. podophyllotoxin, for a period of approximately one hour, some of the first maturation metaphase asters, spindles and chromosomes looked normal, but in others, there was a range of abnormalities, from a slight decrease in size of asters and spindles to complete disappearance of these structures. By 27 minutes after the beginning of treament loose chromosome vesicles were present in the cytoplasm of some eggs, and by 53 minutes after the initiation of treatment, this condition held for all the eggs. No further development occurred.

The less concentrated treatment of 0.00005 mg./ml. resulted, at four minutes after the beginning of treatment, in a reduction in size of maturation spindles and asters in certain ova, while in others, these were essentially normal. By the time of the second maturation metaphase there was a slight reduction in size of the spindles and asters. Subsequently, there was considerable delay in development, as compared with the controls, so that in samples fixed 115 minutes after the beginning of treatment, the experimental eggs were in the 4-cell stage, while the controls were in advanced stages of cleavage (about 16 cells). The resulting experimental trochophores were abnormal, with the characteristic atypical features described above as resulting from podophyllin treatment. Thus, at this low concentration, podophyllotoxin has only slight effects on the spindle mechanism, including a decrease in size; a delay in cleavage occurs, and subsequent development is atypical.

TABLE I

The comparative effects of podophyllin and of podophyllotoxin on the fertilized eggs of Chaetopterus

Conc. and Drug	Effect
0.00001–0.00005 mg./ml. podophyllin	Multipolar figures; abnormal trochophores. Karyokinesis without cytokinesis
0.00005 mg./ml. podophyllotoxin	Reduction in size of asters and spindles; delayed cleavage: 4-cells in exp.; advanced clvgs. in controls
0.0005 mg./ml. podophyllin	Reduction in size of asters and spindles; no polar bodies given off;
0.0005 mg./ml. podophyllotoxin	Very abnormal cleavages at first; some recovery, but not beyond 2-cell stage
0.001 mg./ml. podophyllin	One or two polar bodies present @ 35 abt*; no fusion of pronuclei. Mostly dead by 39 mins. after end of treatment
0.001 mg./ml. podophyllotoxin	Chromosomes in "membrane"; no further development
0.01 mg./ml. podophyllin	Chromosomes within "membrane"; no further develop.
0.01 mg./ml. podophyllotoxin	Chromosomes within "membrane"; no further develop.

All treatments begun 5 minutes after insemination.

* "Abt" = minutes after the beginning of treatment.

The effects of increasing duration of exposure, at the same dosage level, on Chaetopterus eggs treated with podophyllotoxin

To test the effects of increasing the duration of exposure of *Chaetopterus* eggs to podophyllotoxin at a single given concentration, eggs were exposed by the usual techniques to a dosage of 0.0005 mg./ml.; as noted in the preceding section, this treatment could be expected to produce a predictable and reasonably uniform set of results (containment of the chromosomes in a membrane, with subsequent release) when treatment was continued for 60 minutes. In the present series, treatments ranged from 5 to 20 minutes in length.

After the 5-minute treatment (which, like the 10-, 15- and 20-minute treatments, was followed by two washes with large amounts of freshly filtered sea water, to remove the mitotic poison), there was a good deal of recovery, but the eggs were not entirely normal. At the earlier stages after the initiation of treatment, reduction in the size of maturation spindles was noted, as well as some incidence of multipolar figures in the cleavage stages. The 10-minute treatment resulted in considerable variation in effects; these ranged from the presence of thread-like masses of chromatin (sometimes with a suggestion, only, of asters nearby in the eggs fixed 55 minutes after the initiation of treatment, at a stage when the controls were in ±4-cell stages, to fairly normal-looking ±12-cell stages at 175 minutes after the beginning of treatment. In general, it can be said that this treatment resulted in retardation of development and in more marked abnormalities than the 5-minute treatment, but some degree of recovery did occur.

A 15-minute treatment resulted in surface excrescences, of various sizes, simulating cleavage blastomeres, in samples fixed 100 minutes after the beginning of treatment; in some of these pseudo-blastomeres, small abnormal spindles and/or asters were present, in others they were absent. Similar surface excrescences have been reported by Ormsbee *et al.* (1947) for podophyllin-treated mouse tumor cells in tissue culture, by Cornman and Cornman (1951) for podophyllin-treated echinoderm eggs, and by Makino and Cornman (1953) for mouse tissues treated *in vitro* with podophyllotoxin. Thus only a very slight degree of recovery occurred after a treatment of even this short duration, and similar results were observed in eggs treated for 20 minutes. In general, the effects of these shorter exposures to a moderate dilution of podophyllotoxin were comparable to those observed after treatment with lower doses for longer periods.

Five-minute treatments with various concentrations of podophyllotoxin

Three different concentrations of podophyllotoxin (0.001, 0.0003 and 0.0005 mg./ml.) were tested for their effects on *Chaetopterus* eggs after treatments only 5 minutes in duration. At the highest concentration, the cytological picture characteristic of podophyllin and podophyllotoxin treatments was evident at least as soon as 15 minutes after the onset of treatment (10 minutes after the end of treatment); asters and spindles had disappeared and the chromosomes had already taken on a somewhat vesiculated appearance. (The first samples were not fixed until 15 minutes after the beginning of treatment, so we are not able to state how much earlier the characteristic effects were apparent. However, this concentration of podophyllotoxin, in tests over longer periods, resulted in the usual cytological picture very soon after the initiation of treatment.) No further development occurred. After treatment with 0.0003 mg./ml., maturation was completed normally, and a semblance of cleavage occurred, although it was slightly delayed, as compared with the controls. Other eggs from the same batch, which were left in the experimental solution for as long as 20 minutes as a second type of control (in addition to eggs in plain sea water), showed no signs of recovery, and the cytological effects of podophyllin and podophyllotoxin noted above were seen. With an intermediate concentration (0.0005 mg./ml.) the first indication of an effect was in samples fixed 36 minutes after the beginning of treatment, in the first cleavage, where the spindles and asters were smaller than normal. In samples fixed 60 minutes after the beginning of treatment, there were many uncleaved eggs, some with two fairly normal (but small) spindles, others with a single multipolar spindle. None was observed to have progressed beyond the two-cell stage. However, between that time and 175 minutes after the beginning of treatment, when another sample was fixed, some degree of recovery appeared to have taken place, because there were a good many fairly normal advanced cleavage stages; they developed into swimming forms which exhibited various types of abnormality in ciliation, surface blebs, etc., as is characteristic of trochophores developing from podophyllin-treated eggs. Ova which were left in the podophyllotoxin solution for 175 minutes did not develop farther than the usual stage found after relatively prolonged treatment with these antimitotic agents. It appears, then, that eggs can recover to some extent from a 5-minute treatment with this intermediate concentration of podophyllotoxin, but they are not entirely normal.

Treatment of Chaetopterus eggs with mitomycin C, N-dichloroacetyl DL serine and quercetin

In another series of experiments, the possible antimitotic effects of several other agents were tested on fertilized *Chaetopterus* eggs; these agents included mitomycin C,[3] N-dichloroacetyl DL serine (sodium) and quercetin, the last-named substance being a pigment component of crude podophyllin resin which has been reported by Cornman and Cornman (1951) to retard division of echinoderm eggs, but to have no effect in destroying the achromatic figure. The general methods followed in these experiments were the same as those described above for podophyllin and podophyllotoxin treatments.

Mitomycin C was shown by Merz (1961) to induce chromosome breaks and inhibition of mitosis in root tip chromosomes of *Vicia faba*, and by Matsumoto and Lark (1963) to block DNA synthesis in bacteria. In concentrations of 0.002 mg./ml. or 0.0001 mg./ml. this substance did not affect the division of *Chaetopterus* ova, nor their subsequent development, even when the treatment was continued for an hour or longer.

N-dichloroacetyl DL serine, reported by Levi *et al.* (1960) to cause regression of sarcoma 37 in mice, had no effects, either cytological or developmental, at any of the concentrations tested (1–0.005 mg./ml.), except that there were some ciliary defects in a few of the experimental larvae observed the day following treatment.

Quercetin, even in a concentration as high as 1 mg./ml., resulted in essentially normal trochophores and in no observed cytological abnormalities in samples fixed 5, 32 and 55 minutes, and three hours after the beginning of treatment. There was, however, a slight retardation of cleavage.

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[3] We are indebted to Dr. Joel Flaks for his assistance in these experiments.

DISCUSSION

There are, as Biesele (1958) has pointed out, a number of different places in which interference with one process or another may lead to the production of mitotic abnormalities or complete cessation of cell division. However, the opportunities for obtaining abnormalities as a result of an interference with mitosis in eggs or cleavage blastomeres may be considerably greater than in relatively undifferentiated tissue culture cells.

For normal embryonic development, there has to be a correlation between mitotic events and events such as oöplasmic segregation which lead to differentiation. Within the mitotic events, there is, of course, a coordinated relationship between karyokinesis (chromosomal activities), cytokinesis, and normal centriole replication, plus the axial relations of spindle orientations which must be coordinated with the segregation of cytoplasmic constituents destined to become incorporated into particular cleavage blastomeres. If some mitotic poisons affect the chromosome replication or behavior, others the furrowing, still others centriole replication, and still others cell respiration and metabolism, the possibility for the abnormal distribution of cytoplasmic stuffs becomes so great that the chance of obtaining normal development is practically non-existent.

Cytological effects of podophyllin and podophyllotoxin on other materials

The following table is of interest as a comparison of the effective doses of podophyllin and podophyllotoxin, respectively, required to block cleavage in fertilized marine eggs, as reported in this paper and by Cornman and Cornman (1951):

Chaetopterus	Arbacia**	Echinarachnius**
Podophyllin: 10 mg./L.*	6 mg./L.	0.5–1 mg./L.
Podophyllotoxin: 1.0 mg./L.	0.6 mg./L.	0.02–0.04 mg./L.

* For purposes of comparison, our dosages have here been expressed as mg./L., rather than as mg./ml., as we have done elsewhere in this paper.

** Data of Cornman and Cornman (1951).

It is apparent that the *Chaetopterus* egg is somewhat more resistant to the effects of podophyllin than is the egg of *Arbacia*, and very much more resistant than the egg of *Echinarachnius* (which is notoriously sensitive to any adverse condition, such as a slight increase in temperature). Similarly, a podophyllotoxin concentration of 1.0 mg./L. is required to block *Chaetopterus* eggs, while slightly more than half that amount suffices to block *Arbacia* eggs, and even smaller amounts block *Echinarachnius* eggs. This illustrates again the greater efficacy of the active principle of podophyllin as compared with the crude resin.

MacCardle (1951) found that *in vivo* treatment of mouse sarcoma 37 with N-acetyliodocolchinol methyl ether produced anastral spindles. In living cells (teased preparations), the spindle fibers were not visible, but in Heidenhain iron haematoxylin-stained sections, fragments of fibers were present. There were many multipolar mitoses. Low doses of podophyllin (20 micrograms) produced effects on sarcoma 37 cells similar to those found after acetyliodocolchinol treatment. After microincineration of cells treated with either agent, the spindle area was marked by the presence of large masses of white ash of calcium or magnesium; these were much larger than similar masses present in untreated microincinerated cells. Biesele (1958) points out that this white ash might represent divalent ions which had united with a lipoidal constituent of the spindle liberated by action of the mitotic poisons. He also suggests that if the white ash originated in calcium or magnesium from the chromosomes, this might indicate a damaging effect of the poisons on the chromosomes themselves.

A comparison of the effects of podophyllin with those of colchicine[4]

Inoué (1952), using polarization optics, has studied the effects of colchicine, in concentrations from 1×10^{-5} to 1×10^{-2} *M*, on the first maturation spindle of the *Chaetopterus* egg. He observed that the spindles in such treated eggs began to disappear within a very few moments after the initiation of treatment, and his Plate II shows that this effect is first apparent in a diminution in size of the asters at 3 minutes 15 seconds after the beginning of treatment with 5×10^{-4} *M* (0.2 gm./L.) colchicine. With higher concentrations, the effects are apparent sooner and are complete, with disappearance of the spindle, by 4 minutes 5 seconds after the beginning of treatment with 5×10^{-3} *M*. In general, there was a direct relation between the concentration of colchicine used and the time required for complete disappearance of the spindle.

Soon after the beginning of treatment with colchicine in Inoué's experiments, the characteristic birefringence of the astral rays and continuous fibers of the spindles began to decrease, while the spindle length shortened. As this process of spindle shortening continued, loss of birefringence became more pronounced. The length of the spindles at the time when birefringence disappeared seemed to depend upon the concentration of colchicine used, but in all cases he noted, as did we in the present experiments with podophyllin, that the chromosomes remained oriented on the equatorial plate until the spindle had completely disappeared. He observed that the chromosomes then began to scatter in the egg cytoplasm; this is in contrast to our findings in podophyllin-treated eggs, and he does not describe any process of containment of the chromosomes within a membrane, such as we found. As the chromosomes began to move inward from the egg periphery in his studies, a characteristic bulge was observed at the periphery of the egg above them; he interprets this as an abortive polar body, presumably comparable to the similar phenomenon described above for podophyllin-treated eggs. With low concentrations of colchicine, Inoué found that the spindle contracted very slowly; the polar regions began to "disintegrate" and as many as seven parallel "spindles" (his quotation marks) were present, each with a pair of chromosomes at its center. We found nothing comparable to this effect in our studies.

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[4] An important contribution on the mechanism of colchicine inhibition of mitosis by E. W. Taylor (*J. Cell Biol.*, **25**: No. 1, Part II, 145–160; 1965) appeared while this paper was in press. H^3-colchicine was shown to have no direct effects on the duration of the cell cycle (of strain K. B. cultured human cells) or on macromolecular (DNA, RNA, and protein) synthesis, at a concentration of colchicine which completely inhibited mitosis. An exposure of 6 to 8 hours at 10^{-7} *M* was sufficient to block essentially all the cells in metaphase, thus indicating that colchicine is bound to the majority of interphase cells. The data are in agreement with the idea of a mechanism involving reversible binding of colchicine to a set of cellular sites, and suggest that if a critical fraction (3% to 5%) of the sites is complexed, the cell is unable to form a functional mitotic spindle. Presumably, a higher concentration of colchicine would be required to disrupt the mitotic spindle than to prevent its assembly.

The effects of colchicine at the concentrations tested were apparently reversible in Inoué's studies. Eggs treated for 5 and 10 minutes with 10^{-4} *M* mitotic poison were washed in three changes of fresh sea water; at this time the spindle had disappeared and pseudo-polar body formation had taken place. This condition persisted for more than an hour, at which time the polar bulge receded and a small spindle and asters began to appear. In about 3½ hours this spindle had grown to approximately the normal size, and the egg could be successfully inseminated with ensuing polar body formation.

Inoué (1952) interprets his data to indicate that the action of colchicine is to disorganize the orientation of the micelles in astral rays and spindle fibers, most probably by breaking down some chemical bond in or between the micelles and simultaneously causing some of the remaining micelles to contract (as well as breaking down some of the remaining linkages between them). He suggests that colchicine antagonizes the action of some cell component which keeps the spindle substance polymerizing, and which maintains the spindle micelles in their extended form so that they cannot dissociate from one another.

A comparison of the effective concentration of podophyllotoxin (*ca.* 1.0 mg./L.—equal to 2.4×10^{-6} *M*) with the effective dosages of colchicine, used on marine eggs by various authors (see Biesele, 1958, for references), indicates that the former is between 100 and 1000 times as effective in preventing cleavage.

The Merck Index (1960) gives the empirical formula of colchicine as $C_{22}H_{25}NO_6$, with a molecular weight of 399.43. Podophyllin is, of course, a mixture of substances, of which podophyllotoxin is the active antimitotic principle. The formula of podophyllotoxin is given as $C_{22}H_{22}O_8$, with a molecular weight of 414.4. There would, then, be relatively little osmolar difference (less than 4%) between milligram/liter solutions of the two substances, colchicine and podophyllotoxin.

Another study utilizing colchicine was that of Gaulden and Carlson (1951), who described the formation of a hyaline globule in colchicine-treated grasshopper neuroblasts. This globule was seen to arise either from the karyolymph of late prophase or from the spindle of metaphase or anaphase cells. When it originated from the spindle, the first sign of an effect was a reduction in the size of the spindle, to about half its initial size; the globule appeared at one side of the chromosome group and was not surrounded by a membrane. Gaulden and Carlson made three points in summarizing their findings: (1) The greater the concentration of colchicine, the greater its effects in destroying or interfering with development of the spindle. (This is in complete agreement with our findings for podophyllin-treated *Chaetopterus* eggs.) (2) The more completely the spindle was developed at the time of its exposure to colchicine, the greater the concentration required to destroy it or prevent its further development. (In our experiments, the spindle was in most cases at the first maturation metaphase, so that we are unable to draw any meaningful comparisons on this point.) (3) A series of changes of orientation in the chromosomes was seen to be directly related to changes in the spindle structure. (We have already commented on this finding above—see section on "The sequence of early events in the cytological effects of podophyllin.") Gaulden and Carlson (1951) suggest that colchicine does not destroy the spindle material in grasshopper neuroblasts, but merely alters its molecular configuration, so that it becomes a spherical mass with no mitotic function.

The role of the achromatic figure in mitosis

Hiramoto (1956) reported that in fertilized *Clypeaster* eggs at the dumbbell stage, the mitotic spindle and asters could be completely removed, using a micropipette, and furrowing would continue so that most eggs divided in two. If the spindle were removed in the anaphase or early telophase, division continued in some eggs, while in others the furrow receded. In some cases, the initiation of furrowing was seen after both spindle and asters had been removed as early as the metaphase. The cleavage plane was not affected by the removal of the spindle in Hiramoto's studies, although the speed of furrowing was somewhat slower than normal. He demonstrated, also, that the cleavage plane was unmodified when the position of the mitotic figure was displaced during anaphase or later, by removal of a part of the protoplasm; thus, the cleavage plane is already fixed in the egg cortex.

The effects of colchicine on *Psammechinus* eggs were used by Swann and Mitchison (1953) as a tool to test their hypothesis that the asters are only passive guides for the advancing cleavage furrow. They used the poison to suppress the spindles and asters at a time when the chromosomes had already separated, to see if cleavage would ensue; it did so, even in cases where the asters and spindles had completely disappeared, if the cell had reached mid-anaphase when treatment was initiated. This is in agreement with Hiramoto's findings.

Rappaport (1961) compressed *Echinarachnius* eggs into a torus (doughnut) shape, and found that they then divided only in the spindle region, producing a binucleated horseshoe-shaped cell. The second division following this resulted in the isolation of two uninucleated cells from the ends of the horseshoe. The bend of the horseshoe was binucleated, but within about five minutes after completion of the two "normal" divisions, a furrow appeared between the polar regions of the two asters in the binucleated cell. Thus, a furrow was completed in a region which had never been in close proximity to a spindle or to chromosomes but which was marked by the presence of the two asters. Rappaport suggests, then, that in normal cells, the position of the cleavage furrow may be determined by the "zone of confluence" of the asters.

Kobayashi (1962) has described cases in demecolcine-treated multinucleated *Mespilia* eggs where furrows entered from the egg surface between the asters of neighboring achromatic figures; he states that such cases were not frequent, but were usually encountered when the nuclei were crowded.

Although they did not originate the idea of chemical evocation of cytodieresis, Cornman and Cornman (1951) have attempted (p. 1479) to explain their results by the assumption that a furrow-organizer is released from the nucleus at the end of prophase and distributed to the equatorial region by the achromatic figure. This furrow-organizer then causes the furrow to form and to progress through the egg. When podophyllin incapacitates the achromatic figure, the furrow-organizer reaches the cortex late, and in an irregular pattern, causing delayed, irregular furrowing. However, the Cornmans (1951, p. 1476) state that furrow activity maintains a relationship with the chromatin and not with the asters. We question this, since furrowing can occur between cytasters in enucleate fragments of marine eggs (Wilson, 1925). We question, also, whether the furrow-organizing substance is released from the nucleus of the mature egg or from the cleavage nuclei at each successive division. It seems more reasonable to assume that the furrow-organizing substance is a cytoplasmic component, possibly derived from the neutral-red-staining granules described by Kojima (1959).

Kojima (1959) has reported that neutral-red-stainable granules appear in the cytoplasm of eggs of three species of Japanese sea urchins (*Hemicentrotus, Temnopleurus* and *Mespilia*); at first these granules are uniformly dispersed in the cytoplasm of fertilized ova, but soon they gather around the mitotic figure and are distributed to the two cleavage blastomeres. Subsequently, they appear around the mitotic figures at subsequent cleavages; no change in the number of granules was found. Similarly, in parthenogenetically activated eggs, the granules (at first dispersed in the cytoplasm) gathered around the monaster or cytaster, and in centrifuged eggs in which the granules were segregated, cleavage occurred only in the blastomere containing the granules. If unfertilized *Temnopleurus* or *Mespilia* eggs were centrifuged into two halves, only the centrifugal half, which contains the granules, could develop further after fertilization. Kojima also treated fertilized eggs of these forms before and after vital staining, testing a number of mitotic inhibitors including colchicine and dinitrophenol; he found that especially under the influence of DNP-inhibition, the appearance of the granules after vital staining was inhibited until the eggs were returned to sea water, the granules then gathered around the aster. The effects of colchicine were less clear-cut, there being only a reduction in the observed number of stained granules in such treated eggs; these granules remained dispersed through the cytoplasm. It is important to note here that colchicine and DNP act as inhibitors of mitosis in two different fashions, colchicine being a spindle-destroyer and DNP being a respiratory poison; the significance of Kojima's findings on this point is thus questionable.

Rebhun (1959) has described methylene-blue- and toluidine-blue-stainable granules in the *Spisula* egg, which move to the asters and subsequently migrate in a fashion which suggests the possibility that they are distributed by the mitotic apparatus.

Zimmerman and Marsland (1960) studied *Arbacia* eggs which had been subjected after fertilization to centrifugation at high force (40,000–50,000 *g*) and high pressure (8000–12,000 pounds per square inch) for periods of two to five minutes. They demonstrated that such treated eggs could furrow long before the normal time, often irreversibly, as a consequence of the action of the combination of the two physical agents (if treatment was begun before prophase) or of centrifugation alone (if treatment was begun later). It was necessary that rupture of two groups of structures have taken place before successful furrowing would occur: (1) the nucleus, and (2) the metachromatic beta granules. Zimmerman and Marsland postulate that the pressure acts by solating the gel structures of the cell, permitting them to break down more readily and facilitating the separation and stratification of the cell's components. They suggest that the furrowing reaction is normally induced by the transport of materials, both nuclear and cytoplasmic in origin, to the two polar regions (spindle poles) of the cell cortex and, perhaps, the mitotic apparatus constitutes the transporting agency. The experimentally-induced reaction, on the other hand, seems to involve only the centripetal pole, and the transport is achieved through the medium of high centrifugal force.

Whatever the source of the furrow-organizer, it is reasonable to assume that in animal cells, there is an accumulation of substance near or in the centrosomal regions, which is transported by the asters to the periphery of the ovum. This streaming would create a fountain movement, of the type described by Spek (1918) for the egg of *Rhabditis,* and by Conklin (1902, 1938) for the egg of *Crepidula,* with streaming from each aster to the cortical equatorial region (equator of the spindle axis) and then back toward the middle of the egg. The furrow as a constricting ring is initiated at the region of convergence of the fountain streaming. With the reduction or destruction of the asters by podophyllin (or by podophyllotoxin), the furrowing (cytokinesis) is inhibited or prevented. Destruction of the spindle prevents separation, etc., of daughter chromosomes (*i.e.,* karyokinesis).

There is nothing, in its molecular structure, to account for the much greater efficacy of podophyllin, as compared with colchicine, but this probably reflects our lack of knowledge of the structure of the mitotic spindle and the astral radiations. We incline toward the view that podophyllin has a direct effect on these structures, and that interference with the passage of some furrow-forming substance through the asters to the furrow region of a dividing cell is a secondary, rather than a primary, effect. The evidence we have presented in this paper supports such a conclusion.

Summary

1. Fertilized eggs of the polychaete annelid, *Chaetopterus pergamentaceus,* were treated with various dilutions of podophyllin and podophyllotoxin, beginning five minutes after insemination. Observations were made on the living eggs and on whole-mount cytological preparations made from samples fixed at various intervals during development. Dosages ranging from 0.1 to 0.00001 mg./ml. were tested.

2. Beginning as soon as 60 seconds after the beginning of treatment with moderate to high dosages (0.1 to 0.005 mg./ml.) of podophyllin, the asters of the egg maturation figure began to fade, followed by disappearance of the spindle by about three minutes after the initiation of treatment. The nine egg chromosomes remained in the ring configuration (characteristic of the first maturation division in this form) for approximately two to five minutes longer (depending on the exact dosage), after which they were gradually enclosed in a membrane-surrounded area which appears to have been derived from the spindle substance. By this time they had taken on a somewhat vesicular appearance. The sperm chromosomes were often similarly affected by the podophyllin, although the onset of these effects was usually somewhat slower than for the eggs. Both egg and sperm chromosomes

remained contained in this membrane for approximately an hour, after which they were released to lie loose in the egg cytoplasm. No further development occurred at these dosages.

3. After treatments with podophyllin concentrations of 0.00005 to 0.00001 mg./ml., a semblance of normal development ensued, although the resulting larvae were usually abnormal. In egg-samples fixed 24 minutes after the beginning of treatment, there was occasional evidence of reduction in size of the asters; by 47–57 minutes after the initiation of treatment, multipolar figures were observed, as well as two entirely separate complex metaphases, often in uncleaved cytoplasm. There was frequently karyokinesis without accompanying cytokinesis. When cleavage continued to occur, it was often retarded and/or abnormal.

4. Shape changes ("pear" and polar lobe stages) characteristic of the normal development of *Chaetopterus* eggs were observed, even after treatment with high dosages which destroyed the achromatic figure. Pseudo-polar bodies, usually without chromatin, were also noted, as well as giant polar bodies.

5. The effects of podophyllotoxin were qualitatively much like those of podophyllin, but the dosage required to produce them was very much lower (0.001 mg./ml. for podophyllotoxin *vs.* 0.01 mg./ml. for podophyllin to completely block division, for example).

6. Even short durations of exposure to either podophyllin or podophyllotoxin (10–20 minutes, as compared with the 60-minute duration routinely used in the other experiments) were sufficient to induce marked abnormality with moderate dosages.

7. The effects of mitomycin C, N-dichloroacetyl DL serine (sodium) and quercetin were also tested. None of these agents was effective in producing cytological abnormality in *Chaetopterus* eggs, at the concentrations tested.

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THE INFLUENCE OF LIGHT ON THE TIME OF CELL AGGREGATION IN THE DICTYOSTELIACEAE

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Light has long been known to influence the form and dimensions of the fructifications, or sorocarps, of the Dictyosteliaceae. Both Potts (1902) and Olive (1902) reported positive phototropism in the developing fruiting structures of *Dictyostelium mucoroides*, and Potts further observed that fructifications developed in light were smaller than those produced in darkness. Harper (1932) and Raper (1940) reported the latter phenomenon for *Polysphondylium violaceum* and *Dictyostelium discoideum*, respectively. The high sensitivity to light of the migrating pseudoplasmodia of *D. discoideum* was first reported by Raper (1940), and has been further investigated by Bonner *et al.* (1950) and Francis (1964). The object of our study has been to investigate the effect of light on still earlier stages of development, namely, the vegetative and preaggregative stages, with special emphasis on the beginning of cell aggregation in *D. discoideum*.

As early as 1940, Raper noticed that the myxamoebae of *Dictyostelium mucoroides* and *D. discoideum* aggregated earlier if grown in light than if grown in darkness. More recently, Shaffer (1958) found that cells of *D. discoideum* aggregated in bursts after they were transferred from darkness to light; the maximal number of aggregations developed three to four hours after the myxamoebae were illuminated. He further observed (1961) that in *P. violaceum* the rate of center development increased rapidly after plates containing preaggregative myxamoebae were exposed to light for ½ to 1 hour.

In our attempts to elucidate the influence of light on cell aggregation, primary emphasis was given to the type strain of *Dictyostelium discoideum*, but three other strains of this species were investigated as well after preliminary experiments revealed that not all isolates respond equally to identical patterns of dark and light incubation. Myxamoebae of four additional species of the Acrasieae were then grown and tested under different conditions of light and darkness. Finally, an effort was made to determine if cells of *D. discoideum* grown in the light could influence the time of aggregation of cells grown in darkness.

MATERIALS AND METHODS

Cultures examined included several strains of *Dictyostelium discoideum*, representing haploid and diploid cultures of NC-4 (the type) and three other haploid strains (TO-9, WS-7 and Acr-12), *D. mucoroides*, F-15a, *D. purpureum*, WS-321, *Polysphondylium violaceum*, P-6, and *P. pallidum*, WS-320. The myxamoebae were usually grown on a rich peptone-glucose agar medium of pH 6.0 (Bonner,

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1947) in association with *Escherichia coli* #281, *E. coli* B/r, or *Aerobacter aerogenes* #900. They were then harvested at the preaggregative stage and suspended in cold distilled water or in Bonner's salt solution (Bonner, 1947). Excess bacteria were removed by 3 to 5 centrifugations at a relative centrifugal force of 110 *g*. One hundred-μl. drops of a reconstituted suspension adjusted to 4×10^5 cells/ml. were deposited on non-nutrient agar in dim fluorescent light. Three aliquots of the suspension were implanted in each of two Petri dishes to yield six replicate populations with a cell density of *ca.* 200 myxamoebae/mm.[2] (Sussman and Noël, 1952). The dishes were kept uncovered until the excess water had evaporated or had been absorbed by the agar. The test populations were incubated at 23 ±1° C.

Myxamoebae were also grown in shaken cultures according to the technique of Gerish (1959): aliquots of 3 ml. of a bacterial suspension in 18×150 mm. test-tubes were inoculated with spores, grown at 25° C. on a rotary shaker at 250 rpm, and harvested in the vegetative or early preaggregative stage at a density of 3 to 5×10^6 myxamoebae/ml. (Hohl and Raper, 1963).

When populations of myxamoebae were grown in the light on solid media, or were moved to the light after deposition of the 100-μl. drops, the Petri dishes were placed under General Electric "cool white" fluorescent tubes with a spectrum from 400 to 700 mμ and a maximal peak at 580 mμ. The light intensity was *ca.* 60 foot candles at the level of the agar plates in which the myxamoebae were grown, or in which they were incubated after their deposition on solid medium, and *ca.* 300 foot candles at the surface of the tubes in which cells were grown in shaken cultures. Observations were made at hourly intervals as the myxamoebae approached the onset of aggregation.

RESULTS

1. *The effect of an initial dark period on the time of aggregation*

The first experiments were centered upon *Dictyostelium discoideum*, NC-4(H), a haploid strain derived from the diploid stock culture, NC-4. Myxamoebae were grown in darkness on the solid medium. The cells were harvested in the preaggregative stage, centrifuged to remove bacteria, and resuspended as described above. After the myxamoebae were deposited on non-nutrient agar, the plates were incubated for increasing intervals in darkness and then transferred to light. The time of aggregation was defined as the moment when streams directed to a continuing center were clearly visible.

Figure 1 reveals that cell aggregation was delayed in both constant light and constant darkness; and that an initial dark period of 7 to 9 hours, followed by an exposure of from 4 to 2 hours to light, resulted in the earliest aggregation, *i.e.*, 11 to 12 hours after the myxamoebae were deposited on non-nutrient agar. In occasional experiments, not shown in the graph, aggregation occurred after 6 hours darkness followed by 4 hours light. If the initial dark period was extended beyond 7 to 8 hours, aggregation was gradually postponed until the cells reached an age where they would have aggregated if kept in constant darkness. Early aggregation could still occur if the initial dark period was subminimal, but the number of such aggregations per Petri dish was limited to one, or a very few, and the majority of the myxamoebae did not come together until about two hours later. Populations of myxamoebae were recorded as being in the aggregative stage only if the total of developing aggregations in the three populations in a single Petri dish was ten or more.

The division between a subminimal and an optimal initial dark period was often quite sharp, and aggregation normally took place at about the same time in all populations of the duplicate plates, or the appearance of denser areas of cells indicated that aggregation was imminent. In other experiments, however, the time of aggregation of cells incubated under similar light conditions varied by as much as 2 to 3 hours. This was particularly true when the initial dark period was long enough for early aggregation to occur in some cases but too short for others. The exceptional variation seen in the time of aggregation for populations exposed to a 7-hour dark period may have been due, in large part, to differences in the physiological states of the cells that were harvested from the solid medium and used in the separate experiments (Fig. 1). If the myxamoebae were pregrown in liquid shaken culture, the time at which aggregation took place after deposition on agar was similar to or slightly earlier than for populations of cells grown on the solid medium.

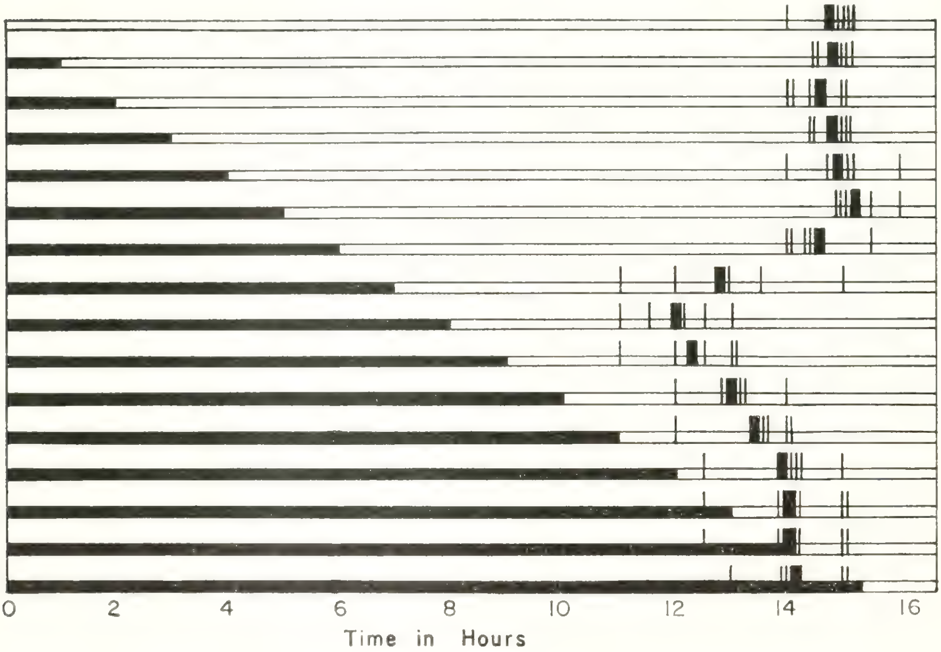

FIGURE 1. The influence of an increasing initial dark period on the onset of aggregation in *Dictyostelium discoideum*, NC-4(H). The graph represents five experiments in which a dark period of *ca.* 8 hours was optimal for early aggregation. The myxamoebae were grown in the dark on the solid medium. Five experiments. ☐ Incubation in light; ■ Incubation in darkness; | Onset of aggregation in individual experiments; ▮ Average time for the onset of aggregation.

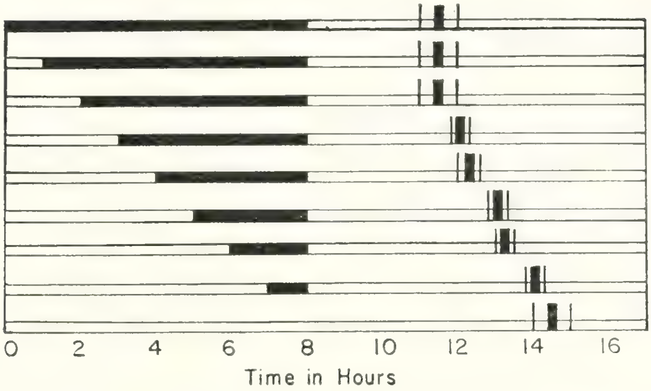

FIGURE 2. The effect of a decreasing dark period on the onset of aggregation in *Dictyostelium discoideum*, NC-4(H), when the transfer to light takes place 8 hours after the deposition of the myxamoebae on non-nutrient agar. The cells were grown in the dark on the solid medium. The graph represents two experiments. ☐ Incubation in light; ■ Incubation in darkness; | Onset of aggregation in individual experiments; ▮ Average time of aggregation.

2. *The effect of later, short dark periods on aggregation*

Myxamoebae were deposited on non-nutrient agar, incubated first in the light for varying periods of time, then transferred to darkness. After a total of 8 hours, all plates were returned to the light. A dark period of at least 4–6 hours had to precede

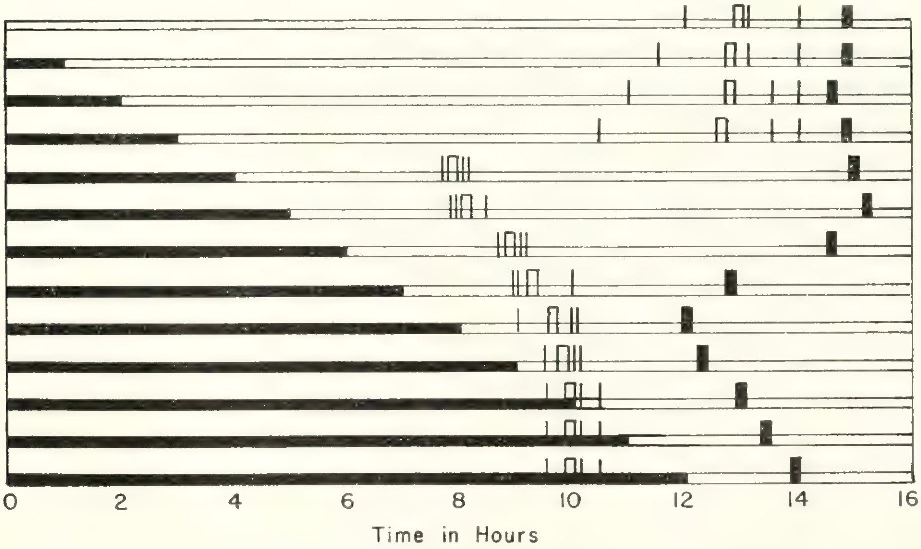

FIGURE 3. The influence of an increasing initial dark period on the onset of aggregation in *Dictyostelium discoideum*, NC-4(H). The myxamoebae were grown on the solid medium. ☐ Incubation in light; ■ Incubation in darkness; | Onset of aggregation of myxamoebae that were grown in the light (three experiments); ▯ Average time of aggregation of cells grown in the light; ▮ Average time of aggregation of myxamoebae that were grown in the dark (data of Fig. 1).

the transfer to light in order to obtain aggregation within a 12-hour period (Fig. 2). A gradual decrease of the dark interval resulted in a progressive delay in the onset of aggregation.

Not only does light affect the time of aggregation after myxamoebae are harvested and deposited on agar, but it also exerts an influence when applied during their growth phase. The results of three representative experiments are shown in Figure 3. Myxamoebae were grown on agar under cool fluorescent light but were exposed to increasing dark periods after being harvested and deposited. The optimal dark period for early aggregation was reduced to *ca.* 4 hours, whereas dark periods of 3 hours or less did not accelerate aggregation significantly. When the initial dark period was increased above 4 hours, the time of aggregation was gradually delayed. In constant dark, and more particularly in constant light, cell aggregation was postponed still further. Under all test conditions, the cells grown on the agar medium in the light aggregated earlier than myxamoebae that were similarly grown in darkness. Comparable results were obtained for cells grown in liquid cultures as for those grown on the solid medium.

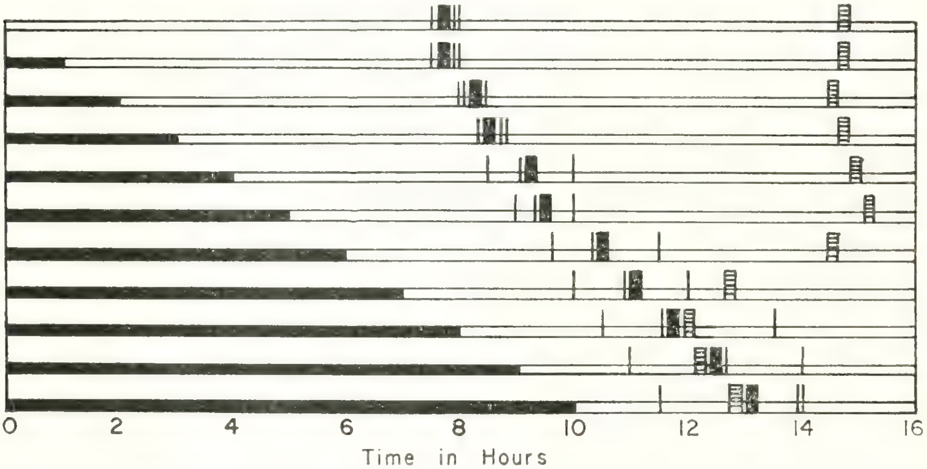

FIGURE 4. The influence of an increasing initial dark period on the onset of aggregation in *Dictyostelium discoideum,* Acr-12. The myxamoebae were grown in darkness on a solid medium. ☐ Incubation in light; ■ Incubation in darkness; | Onset of aggregation in *D. discoideum,* Acr-12 (three experiments); ▌ Average time of aggregation in *D. discoideum,* Acr-12; ☰ Time of aggregation in *D. discoideum,* NC-4(H) (data of Fig. 1).

3. *Light requirements for optimal aggregation in different strains of the same species*

Conditions of dark and light incubation similar to those optimal for *D. discoideum,* NC-4(H), were required also for early aggregation in strains NC-4 (Stock), NC-4(S1) and NC-4(S2), TO-9, and WS-7. The times of aggregation under various light conditions were analogous to those shown in Figure 1. *Dictyostelium discoideum,* strain Acr-12, however, deviated remarkably from this pattern (Fig. 4). Contrary to its effect on other strains, constant light was optimal for early aggregation in this case; an increased delay in aggregation paralleled an increase in the dark period, and the longest delay took place in constant darkness.

4. *The effect of light on aggregation in other species of the Dictyosteliaceae*

The influence of different light conditions on the time between deposition of the myxamoebae and the onset of aggregation in species other than *Dictyostelium discoideum* was first studied in *D. purpureum*, strain WS-321. Myxamoebae were grown in the dark both on solid medium and in liquid culture. Aggregation began after 3–4 hours in populations grown on the solid medium and kept in constant light after harvest and deposition on non-nutrient agar (Fig. 5). An increasing dark period caused a progressive delay in aggregation until the cells would have aggregated without any light.

The time of aggregation was dependent not only on light, but also on cultural and environmental conditions. For example, cells grown in shaken cultures usually aggregated earlier than cells grown on an agar medium; and aggregation was delayed if the excess of water in the 100-μl. drops evaporated slowly following deposition.

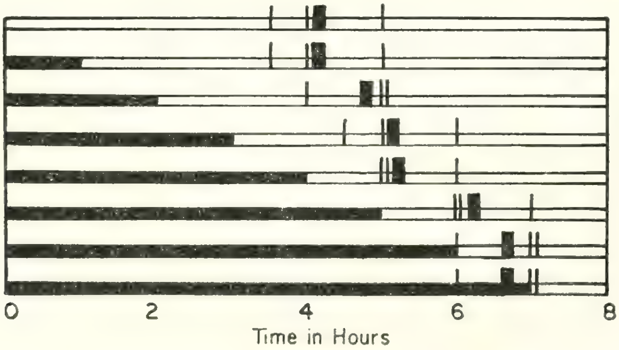

FIGURE 5. The effect of an increasing initial dark period on the onset of aggregation in *Dictyostelium purpureum*, WS-321. The myxamoebae were grown in darkness in shaken liquid culture. □ Incubation in light; ■ Incubation in darkness; | Time of aggregation in WS-231 (three experiments); ▌ Average time of aggregation in WS-321.

Like *Dictyostelium purpureum*, the other species examined, *i.e., D. mucoroides*, F-15a, *Polysphondylium violaceum*, P-6, and *P. pallidum*, WS-320, required constant light for the earliest possible aggregation, while an increasing dark period gradually delayed the onset of this process.

5. *The effect of light-grown cells on the aggregative behavior of cells grown in the dark*

Myxamoebae of *D. discoideum*, NC-4(H), were grown both in light and in darkness in liquid shaken cultures. After harvest and centrifugation, the light-grown cells and the dark-grown cells were mixed in ratios of 1:1 and 1:10. Populations of mixed cells, and of light-grown and dark-grown cells alone, were plated on non-nutrient agar and incubated in constant light, constant darkness, and with initial dark periods of 4, 6 or 8 hours followed by light. The myxamoebae grown in the light aggregated earlier than the cells grown in darkness (Fig. 6). If the light- and dark-grown cells were mixed in a ratio of 1:1 the time of aggregation was intermediate between that of the light and the dark controls, but closer to that of the light-grown myxamoebae. Even if only 10% of the myxamoebae were grown in the light, they still reduced the time of aggregation compared with pure populations of dark-grown cells.

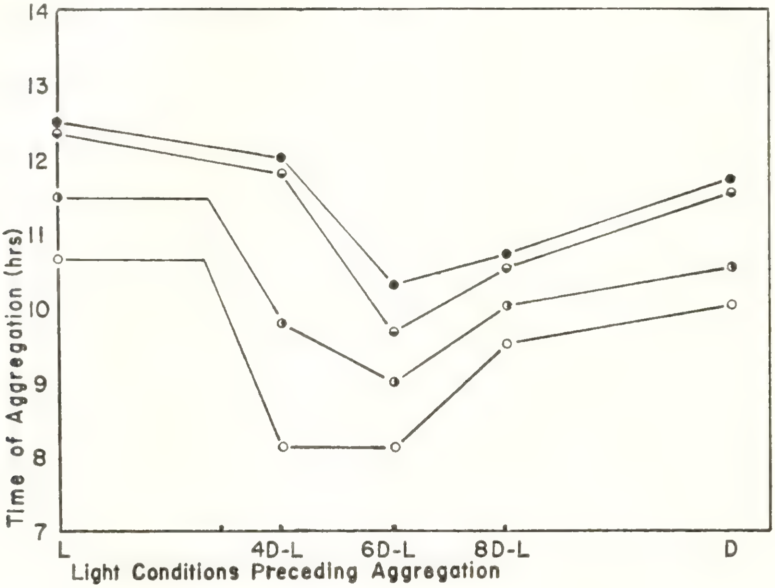

FIGURE 6. The influence of different light conditions on the time of aggregation in *Dictyostelium discoideum*, NC-4(H), in light-grown and in dark-grown myxamoebae and in mixtures of the two; myxamoebae grown in liquid shaken culture. Data represent averages for 3 experiments in each case. *Populations tested*: ○: Cells grown in the light; ◑: Light- and dark-grown cells in a ratio of 1:1; :◒ Light- and dark-grown cells in a ratio of 1:10; ●: Cells grown in darkness. *Patterns of incubation*: L: Continuous light; 4D-L: 4 hours dark followed by light; 6D-L: 6 hours dark followed by light; 8D-L: 8 hours dark followed by light; D: Continuous dark. (The two lower curves are drawn with breaks at 3L-D to reflect data obtained in earlier experiments. See Figure 3.)

DISCUSSION

Two striking features of this study are (1) the dependence of the onset of aggregation on the prevailing light conditions, and (2) the different effects of altered light conditions on different species and even in different strains of the same species. After deposition of the centrifuged cells, constant light was optimal for aggregation in most species, while an increasing dark period gradually delayed the occurrence of aggregation. In *Dictyostelium discoideum* two patterns of aggregation were observed. One strain, Acr-12, behaved in the same manner as *D. mucoroides,* *D. purpureum*, *Polysphondylium violaceum* and *P. pallidum;* other strains of *D. discoideum*, including the species type (NC-4) and both haploid [NC-4(H) and NC-4(S2)] and diploid [NC-4(S1)] substrains derived from it, required an initial dark period followed by constant light for the earliest onset of aggregation. After a subminimal dark period, a few aggregations might form in most strains of this species, but the majority of the cells showed no response to these light conditions and aggregated a few hours later. At a density of about 200 cells/mm.^{2}, neither light nor darkness has a veto-role in the process of aggregation. Together, in the right sequence and proportions, they can stimulate the process but neither can prevent it. Although *Dictyostelium discoideum* seems to be generally less sensitive to the effect of light than other species of the Dictyosteliaceae, it was studied most intensively because of our accumulated experience with this species (Konijn and Raper, 1961).

The light conditions under which the myxamoebae are *grown* before harvesting and centrifugation also affect the time when these can aggregate. Cells grown on a solid medium or in shaken cultures under white fluorescent light aggregate earlier than do cells grown in darkness, and the initial dark period emphasized in Figure 1 is shorter for light-grown myxamoebae than for dark-grown cells.

One might expect that the aggregation time in a mixed population of light- and dark-grown cells would be similar to that of an all light-grown population, or that possibly even a single light-grown cell might stimulate aggregation within a dark-grown population. This, however, is not the case. In fact, the time of aggregation is accelerated only slightly in mixtures of 1:10 light- and dark-grown myxamoebae, and less than 50% in mixtures of 1:1. The time of aggregation in such mixtures must depend, therefore, upon an interaction between the light-grown and the dark-grown myxamoebae.

We wish to thank Miss Judith Jacobsen for her able technical assistance. The work was made possible by research grants from the National Institutes of Health (C-2119), U. S. Public Health Service, and the National Science Foundation (G-3375).

SUMMARY

The time at which aggregation begins in the Dictyosteliaceae was found to be influenced by the conditions of light to which the myxamoebae were exposed during the vegetative and preaggregative stages. The myxamoebae were usually pregrown in the dark on *Escherichia coli* or *Aerobacter aerogenes*, harvested, washed by centrifugation, resuspended, deposited on non-nutrient agar, and incubated under varying regimens of light and darkness. Myxamoebae of the single strains of *Dictyostelium mucoroides*, *D. purpureum*, *Polysphondylium violaceum* and *P. pallidum* included in the tests, and one strain of *D. discoideum* (Acr-12), aggregated earliest if exposed to constant light. Other strains of *D. discoideum*, including the type, NC-4, and substrains derived from it, aggregated optimally after an initial dark period of 6 to 8 hours followed by about 4 hours of light. When these latter strains were *grown* in light rather than in darkness, the initial dark period required for early aggregation was reduced to 4 hours. When light-grown cells of strain NC-4(H) were mixed with cells grown in the dark, the time of

aggregation of the latter was accelerated, but the addition of such cells did not induce dark-grown cells to aggregate as quickly as populations of light-grown cells alone.

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VARIATION IN LINEAR DIMENSIONS, TEST WEIGHT AND AMBULACRAL PORES IN THE SAND DOLLAR, ECHINARACHNIUS PARMA (LAMARCK)[1]

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That the regular sea urchins have considerable variation in respect to a number of meristic and mensural characteristics has long been recognized. In varying degrees Jackson (1912, 1914, 1927), Kongiel (1938), Vasseur (1952), and Swan (1962) have studied this variation and suggested that at least some of it might be either environmentally induced or selected. Kongiel also applied his methods of analysis to fossil remains of heart urchins, and Kermack (1954) and Nichols (1959a, 1959b, 1962) have more recently extended this work. Durham (1955) in his monumental "Classification of Clypeasteroid Echinoids" has indicated that in the sand dollars there is a great deal of variation, and Raup (1958) has gathered evidence that in the Pacific Coast sand dollar, *Dendraster excentricus* (Eschscholtz), heavier tests are characteristic of populations from colder water.

In the present paper findings and suggested possible causal relationships with environmental factors are presented for *Echinarachnius parma* (Lamarck), based on collections from several localities in New England on the Atlantic Coast. The characteristics studied are (1) the relationship between length and width of the test, (2) test weight at comparable sizes, and (3) the numbers of pore-pairs in the petaloid areas of the aboral surface of the test. This material extends some of Durham's (1955) findings and is suggestive of the need for carefully designed statistical studies of variation in this species and for experimental studies on the effects of differing environmental factors on its growth pattern.

MATERIALS AND METHODS

Collections

Six series of sand dollars were collected intertidally, either by hand, or with the aid of a rake. A series of specimens was taken from each of the following places: Crow Neck, North Trescott, Washington County, Maine (44° 52′ 37″ N, 67° 07′ 38″ ±10″ W); Bailey's Mistake, South Lubec, Washington County, Maine (44° 46′ 23″ N, 67° 03′ 16″ W); Paradise Point, Boothbay, Lincoln County, Maine (43° 51′ N, 69° 35′ W); Hampton Beach, Rockingham County, New Hampshire (42° 54′ 07″ N, 70° 48′ 40″ W); Hampton Harbor, Rockingham County, New Hampshire (42° 53′ 59″ N, 70° 49′ 07″ W); and Scusset Beach, Barnstable County, Cape Cod, Massachusetts (41° 47′ 18″ N, 70° 30′ 30″ W).

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[1] This work represents a portion of a dissertation submitted to the University of New Hampshire in partial fulfillment of the requirements for the Ph.D. degree.

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The total number of specimens collected from these six areas was 1696. Two additional series, from New Castle, Rockingham County, New Hampshire (43° 03′ 22″ N, 70° 44′ 17″ W) and Eastern Point, Gloucester, Essex County, Massachusetts (42° 35′ 00″ N, 70° 40′ 00″ W) were collected subtidally. At New Castle a skindiver made the collection from about 5 feet of water while at Gloucester the animals were obtained with a rake over the side of a rowboat. The total number of specimens collected from these two localities was 519. The animals were preserved in 10% formalin in fresh water for 24–72 hours and then dried at room temperature before being measured.

The rake was purchased as a steel garden rake 14 inches wide and about 4 inches high at the opening, with two-inch teeth spaced one inch apart along the lower side of the opening. The handle, 5 feet in length, was made of wood. Hardware cloth ($\frac{1}{4}$-inch mesh) was used to construct a basket, 14 inches deep, which was fastened to the opening between the bow and the rake proper. This basket was attached by means of wire.

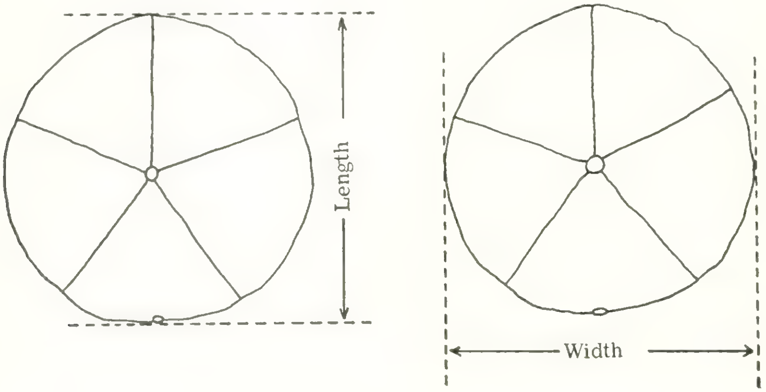

FIGURE 1. Diagrammatic oral surface of the sand dollar, *Echinarachnius parma*, showing length and width of the test.

Method of measuring

A vernier caliper calibrated on the main scale in millimeters and on the vernier to tenths was used for all the measurements. The spines were removed from those portions of the test where contact with the calipers was to be made.

Length

The distance from the edge where the anus is located to the opposite edge of the animal is considered the length of the test (Fig. 1).

Width

The width of the test is obtained by measuring the distance from side to side, perpendicular to that of the length (Fig. 1).

Average diameter

½ (Length + Width), as used by Durham (1955), has been used as the basic criterion of the animals' size.

Variation in Relative Linear Dimensions

The specimens of each series were measured to the nearest tenth of a millimeter for length and width. The resulting data were tabulated into groups for each 5-mm. interval of mean test diameter, ½ (Length + Width). The numbers of specimens longer than wide, having length equal to width, and wider than long have been tallied. Tallies for all eight series were prepared. A summary of these data for all the series, but without reference to size of individuals, is given in Table I. The data tabulated according to size group show no appreciable change in relationship with size. Mean dimensions for the four chief series divided into size groups are plotted in Figure 2.

Table I

Relative lengths and widths of specimens from different localities

Locality	Numbers of specimens			
	Longer	Wider	L = W	Total
Hampton Harbor, N. H.	117 (37.6%)	176 (56.6%)	18 (5.9%)	311
Hampton Beach, N. H.	11 (3.0%)	358 (96.5%)	2 (0.5%)	371
Bailey's Mistake, Maine	23 (7.5%)	282 (92.2%)	1 (0.3%)	306
Crow Neck, Maine	250 (78.9%)	57 (18.0%)	10 (3.2%)	317
Scusset Beach, Mass.	11 (4.5%)	229 (93.1%)	6 (2.4%)	246
New Castle, N. H.	53 (29.4%)	121 (67.2%)	6 (3.3%)	180
Boothbay Harbor, Maine	81 (55.9%)	55 (37.9%)	9 (6.2%)	145
Gloucester, Mass.	112 (32.6%)	211 (61.3%)	21 (6.1%)	344

Examination of these data reveals that the populations from Hampton Beach, New Hampshire, and those from Bailey's Mistake, Maine, have more wider than longer individuals and average wider than long more markedly than is the case for any of the other collections. In distinct contrast to these collections is the one from Crow Neck, Maine, whose members tend to be longer than wide. The collections from the other five localities fall well between these extremes.

Comparisons of the mean dimensions of these five collections (Hampton Harbor, New Hampshire; Boothbay Harbor, Maine; New Castle, New Hampshire; Gloucester, Massachusetts; and Scusset Beach, Massachusetts) reveal that at Scusset Beach the tests grow appreciably wider than long but not as markedly so as at Hampton Beach and Bailey's Mistake. The collection from New Castle averages slightly over 1% wider than long. In none of the other three series (Hampton Harbor, Paradise Point, and Gloucester) is one dimension more than 1% greater than the other dimension. Likewise in none of these series does the ratio of longer to wider specimens deviate from equality beyond 2:1 or 1:2.

The habitat at Crow Neck is unusual. Because it is on the shore of a constricted channel through which large amounts of water pass to and from large inner bays, to the extent of changing their levels many feet on each cycle, it has an almost continuous but reversing flow of water. This resembles a rather swift river which flows nearly six hours in one direction, followed by a brief period of nearly slack water, and then another period of rapid flow in the opposite direction, again for nearly six hours. One would thus expect only rocks and relatively coarse sediments, which are in fact found in the main channels. Most of the sand dollars were found living in eddies which occurred in the small wider parts of the channel. The rather soft sandy substratum under these eddies was generally finer than in the main channels and was often overlain by a layer of mud, silt, or detritus. Thus, the sand dollars living in this habitat live in contact with a nearly continuous current but encounter almost no pounding by waves, and the current's direction changes only a few times a day. It appears that they tend to grow longer than wide.

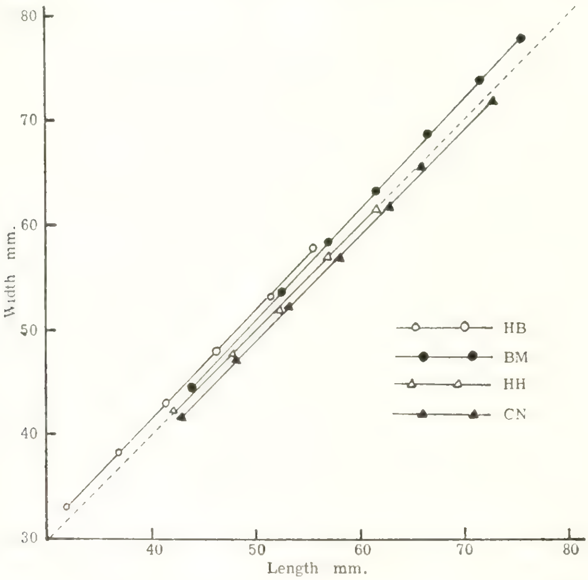

FIGURE 2. Relationship of width to length. HB—Hampton Beach; HH—Hampton Harbor; BM—Bailey's Mistake; CN—Crow Neck.

In contrast with the conditions at Crow Neck we find at Hampton Beach and Bailey's Mistake little continuous current and considerable exposure to surf.

At Hampton Beach, particularly, the surf action is considerable almost all the time. Here the sand grains are fine and hard-packed. The situation at Bailey's Mistake is similar to Hampton Beach but there appears to be some reduction in surf action by the headlands to either side of the entrance to the bay, as is made evident by the eel grass growing in the bay, which in turn appears to give the organisms living among it additional protection. The sand dollars that live in these two places (see Fig. 2) appear to grow quite consistently wider than long.

A situation between the continuously running water at Crow Neck and surf at Hampton Beach can be found at Hampton Harbor. It is a protected harbor southwest of the village of Hampton Beach. Since it is protected, it has little surf action. The current, due to the flow in and out of the tide, is appreciable but much less than at Crow Neck. The area where the sand dollars were collected is covered by water most of the time. During extremely low tides it becomes a shallow quiet pool. The substratum is a mixture of sand and mud with much organic matter. From Table I it can be seen that the animals usually grow a little wider than long. However, they are not nearly as wide as those from Bailey's Mistake or from Hampton Beach. This is true both in terms of average dimensions and in terms of the number of individuals wider than long compared with the number longer than wide.

Thus there are three kinds of habitats involved in this study, namely: (1) soft sand substratum and continuous current, (2) substratum with appreciable amounts of mud, probably high in organic matter, under relatively quiet water with little surf action, and (3) hard-packed fine sand, containing no evident mud and probably little organic matter, associated with heavy surf.

These observations suggest significant correlation between the kind of habitat and the usual form of the sand dollars inhabiting these habitats. Whether the differences in habitat influence the growth pattern of the animals or bring about a differential selection from a heterogeneous gene pool has not been determined.

VARIATION IN TEST WEIGHT

Ten specimens were picked from each 5-mm. size group from the four main series. The spines of these specimens were removed, and then the tests were weighed in grams to the nearest 0.01 gm. The average weights obtained and the average dimensions of the groups of specimens used from the four localities, Crow Neck, Hampton Harbor, Bailey's Mistake, and Hampton Beach, were tabulated.

Obtaining the real test weight, excluding the spines, the soft parts, and the contents of the digestive tract, is a problem. In order to approach a close approximation to the true test weight, one must remove the spines and then cut the test with a thin saw. Raup (1957) used a diamond saw. Then his specimens were cleaned, dried, and weighed to the nearest 0.1 gm.

After he determined the loss of weight (amounting to about 5% for a specimen 35 mm. in diameter) for 24 of his specimens, he considered it insignificant and disregarded it in his computations.

To determine the loss resulting from the cut and the weight of the soft parts and the gut contents, I selected 10 specimens of approximately the same size (*ca.* 50 mm. in mean diameter) from each of the main series.

The spines were removed and the tests were weighed to the nearest 0.01 gm. They were then cut with a thin electric saw and weighed again. The soft parts and the gut contents, but not the lantern, were removed and the weights were determined once more. The total loss of weight, loss from the cut, and the weight of the soft parts and the gut contents were calculated as percentages in the following manner:

$$\text{The total loss of weight} = \frac{\text{The difference in weight before cutting and after cleaning inside}}{\text{Weight before cutting}} \times 100$$

$$\text{The weight loss from the cut} = \frac{\text{The difference in weight before and after cutting}}{\text{Weight before cutting}} \times 100$$

$$\begin{array}{l}\text{The weight of the soft parts}\\ \quad\text{and gut contents}\end{array} = \frac{\text{The difference in weight after cutting and after cleaning inside}}{\text{Weight before cutting}} \times 100$$

The percentage figures are shown in Table II. It should be noted that the lantern is considered part of the test weight. This is in contrast with Raup's (1957) method.

From Table II we can see that the greatest percentage of total loss in weight was 8.7% from a specimen from Bailey's Mistake and the lowest one was 1.9% from a specimen from Hampton Beach, with a mean of 4.14% for all 40 specimens. The weight of the soft parts and the gut contents ranged from 6.1% down to 0.6% among the 40 specimens. It appears that the weight of the soft parts and the gut contents is of little significance and thus has very little effect on the total weight of the dried animal. However, it is interesting to note that the average percentages of the soft parts and gut contents appear to vary with locality and, rounded to the nearest $\frac{1}{2}$%, amount to $1\frac{1}{2}$%, $2\frac{1}{2}$%, 5%, and $1\frac{1}{2}$% from Crow Neck, Hampton Harbor, Bailey's Mistake and Hampton Beach, respectively. Therefore, in this part of the investigation, the weight of the test used, in each series, was obtained by subtracting this predetermined percentage of weight of the soft parts and the gut contents from the specimen's weight before cutting.

The test weights in grams are tabulated and plotted against mean diameters in Table III and Figure 3. As expected, the weights increase slowly when the animals are small in size and then increase somewhat more rapidly as they become larger. In general it appears that at comparable sizes the specimens from Crow Neck are heaviest and those from Hampton Beach are lightest. The series from Bailey's Mistake and Hampton Harbor are between the extremes, with those from Bailey's Mistake being slightly heavier.

Raup (1958) discussed "The Relation between Water Temperature and Morphology in *Dendraster,*" and found the tests of the Pacific sand dollar, *Dendraster excentricus* (Eschscholtz), of a given size to be heavier in cold water than in warm water. He also stated that this correlation is interpreted as the result of phenotypic (nonheritable) adaptation to water temperature. The findings here noted appear to correspond to Raup's ideas. The weights of specimens from Crow Neck and Bailey's Mistake, both from eastern Maine, are heavier than the specimens from Hampton Harbor and Hampton Beach, both from the coast of New Hampshire. According to the "Surface Temperatures at Tide Stations, Atlantic Coast" (U. S. Department of Commerce, 1951), the annual average surface water temperature for Eastport, Maine, from 1944 to 1950 was 44.1° F. and for Portsmouth, New Hampshire, from 1944 to 1950 was 47.4° F. It can

TABLE II

Weight losses incurred by cutting tests and cleaning tests. The deductions from total weights made in Table III are based on these figures

#		Weight			Weight loss %			Ave. soft part & gut contents rounded to nearest ½%
		Before cutting	After cutting	After cleaning inside	Total	Cut	Soft part & gut contents	
CN	121	11.60	11.44	11.33	2.3	1.4	0.9	1.5%
	125	9.95	9.75	9.55	4.0	2.0	2.0	
	130	9.15	8.95	8.80	3.8	2.2	1.6	
	135	5.94	5.84	5.71	3.8	1.7	2.1	
	136	11.69	11.52	11.24	3.8	1.5	2.3	
	137	9.61	9.47	9.36	2.6	1.5	1.1	
	140	9.21	9.10	8.97	2.6	1.2	1.4	
	146	10.10	9.97	9.81	2.9	1.3	1.6	
	152	7.58	7.52	7.38	2.6	0.8	1.8	
	163	8.54	8.43	8.28	3.0	1.3	1.8	
HH	89	7.50	7.30	7.10	5.3	2.7	2.6	2.5%
	94	6.90	6.70	6.45	6.5	2.9	3.6	
	115	8.92	8.81	8.60	3.6	1.2	2.4	
	121	7.57	7.48	7.23	4.5	1.2	3.3	
	123	8.16	8.08	7.91	3.1	1.0	2.1	
	136	8.04	7.92	7.73	3.6	1.5	2.1	
	137	8.17	8.08	7.82	4.3	1.1	3.2	
	154	7.52	7.43	7.24	3.7	1.2	2.5	
	158	7.63	7.54	7.41	2.9	1.2	1.7	
	177	7.64	7.52	7.32	4.2	1.6	2.6	
BM	4	7.45	7.35	6.90	7.4	1.3	6.1	5%
	5	8.81	8.68	8.21	6.8	1.5	5.3	
	7	7.72	7.64	7.16	7.3	1.0	6.3	
	9	8.01	7.89	7.65	4.5	1.5	3.0	
	10	7.45	7.30	6.80	8.7	2.6	6.1	
	12	7.27	7.16	6.78	6.7	1.5	5.2	
	16	6.90	6.81	6.46	6.9	1.3	5.6	
	17	8.94	8.82	8.49	5.0	1.3	3.7	
	19	8.29	8.19	7.78	6.2	1.2	5.0	
	24	7.15	7.06	6.64	7.1	1.3	5.8	
HB	328	7.00	6.80	6.70	4.3	2.9	1.4	1.5%
	329	6.22	6.16	6.05	2.7	1.0	1.7	
	334	7.20	7.13	7.06	1.9	1.0	0.9	
	337	6.56	6.50	6.43	2.0	0.9	1.1	
	339	7.07	6.99	6.90	2.4	1.1	1.3	
	341	5.89	5.83	5.76	2.2	1.0	1.2	
	345	5.08	5.01	4.98	2.0	1.4	0.6	
	346	6.75	6.65	6.50	3.7	1.5	2.2	
	348	4.98	4.92	4.85	2.6	1.2	1.4	
	167	5.82	5.76	5.70	2.1	1.0	1.1	

TABLE III

Mean weights and diameters of specimens. Each average is that of 10 individuals unless a smaller number is indicated in parentheses after the mean diameter figures

Crow Neck		Hampton Harbor		Bailey's Mistake		Hampton Beach	
½ (L + W) mm.	Total wt. −1.5% (soft part) gm.	½ (L + W) mm.	Total wt. −2.5% (soft part) gm.	½ (L + W) mm.	Total wt. −5% (soft part) gm.	½ (L + W) mm.	Total wt. −1.5% (soft part) gm.
14.85 (8)	0.31		–			–	–
20.99 (7)	0.54	–	–	–	–	21.59	0.61
–	–	–	–	–	–	25.96	0.95
31.24 (7)	1.98	–	–	–	–	30.52	1.53
36.51	3.00		–		–	35.40	2.08
40.64	4.12	41.82	3.90		–	40.13	2.77
45.76	6.36	45.48	5.20		–	45.21	4.01
51.55	9.20	51.24	7.54	52.00	7.50	51.69	6.17
55.44	11.41	55.30	8.52	55.36	8.67	56.68	7.39
60.52	12.96	60.63	10.91	60.34	11.34	–	
65.37	15.97	–		65.73	13.70	–	–
72.40 (6)	17.93	–		70.72	16.62	–	–
		–		75.27	18.97	–	–
–		–	–	82.32	23.58	–	–

be seen that the difference in water temperature for this period was 3.3° F. More work needs to be done before any causal relationship can be demonstrated, and the differences found between the two New Hampshire series and between the two Maine series suggest that other factors may be involved.

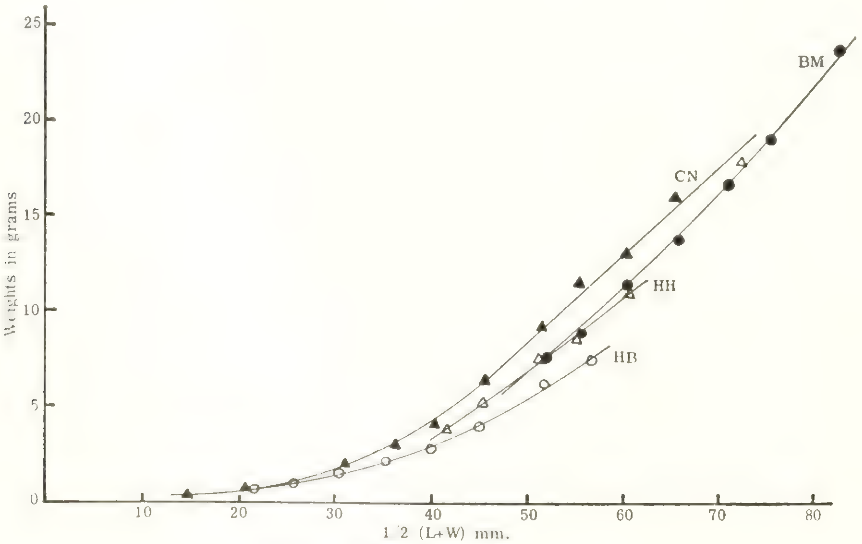

FIGURE 3. Relationship of test weight to size, ½ (L + W).

Variation in Pore-Pairs in Petals

The five smallest specimens of each 5-mm. size group from each of the four main series were used for the counting of pore-pairs in the petaloid areas of the ambulacra. The numbers of pore-pairs in all five petals in each specimen were counted. In order to set up a standard for all specimens, it was necessary to determine the distal limits of the petals. Figure 4 shows that there are two rows of pore-pairs in each, starting at the ambulacral plates nearest the apical system. Near the apex these two rows of pores are close together. Gradually these rows of pore-pairs spread apart and then toward the periphery they come closer together again, but remain separated. Thus, in the terminology of students of the irregular urchins (*cf.* Durham, 1955) *Echinarachnius* has "open petals." Beyond the point of this coming together of the rows of pore-pairs, they diverge, and these conspicuous pore-pairs become infrequent. In this investigation counting of pore-pairs has been stopped at the point where the rows start diverging from each other (Fig. 4).

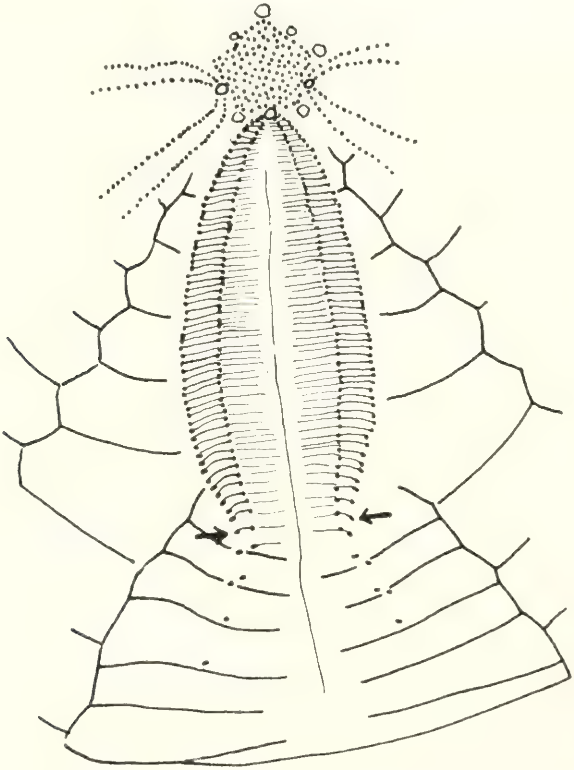

FIGURE 4. An ambulacral sector of the aboral surface of the sand dollar, *Echinarachnius parma,* showing the arrangement of ambulacral pores in the "petal" of ambulacral area II. The arrows indicate the last pore-pairs to be counted in determining the number of these included in the "petal." Specimen from Boothbay Harbor, Maine (3.1 ×).

The mean diameters and numbers of pore-pairs in each size group for each series are summarized in Table IV. There are only two common size groups among these four series.

When we compare the four populations and limit our findings to these two common size groups, it is obvious that, in both size groups, the animals from Crow Neck have on the average the smallest number of pore-pairs in the petals. The specimens from Hampton Beach and Hampton Harbor are close together and compete for having the highest numbers of pore-pairs. The Bailey's Mistake specimens show numbers between these extremes. Because of the differences in the size ranges among these series, it is difficult to compare them directly. To minimize this difficulty, a graph showing the relationship of the number of pore-pairs to size, $\frac{1}{2}$ (L + W), has been drawn as Figure 5.

From this graph it is apparent that among these four series, the series from Crow Neck has the lowest number of pore-pairs whereas the Hampton Harbor series has the highest at comparable sizes. The Hampton Beach and Bailey's Mistake series are in between with Hampton Beach being somewhat higher and at some sizes nearly the same as Hampton Harbor. The lines drawn on the graph have been fitted by eye. With the amount of data presently available, it appears doubtful that more careful fitting would be justified.

Durham (1955, p. 87) states that it appears probable that the number of plates inside the petals is a better indication of the age than is the absolute size. If this is true, the sand dollars that live at Crow Neck are growing faster than those

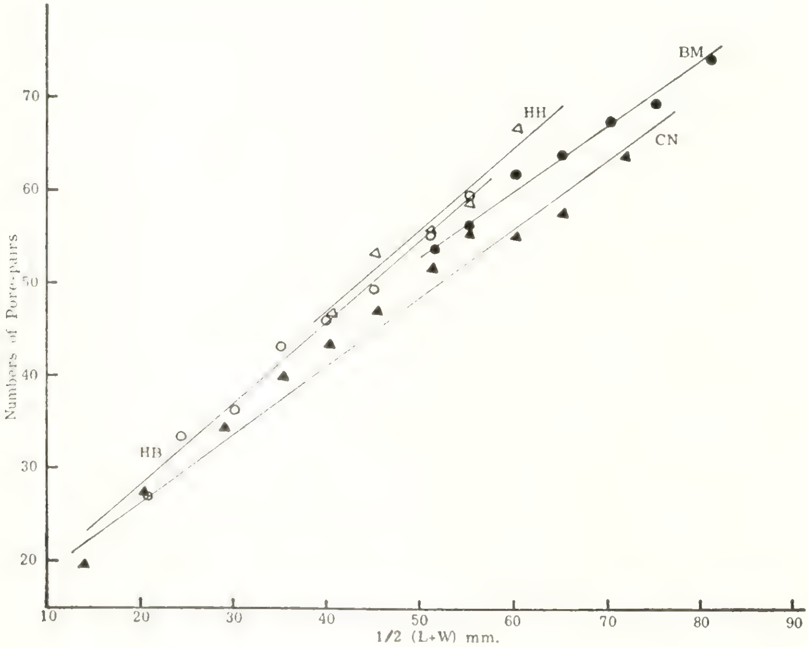

FIGURE 5. Relationship of the number of pore-pairs to size, $\frac{1}{2}$ (L + W).

Table IV

The mean dimensions and pore-pairs. Each average is that of 5 individuals unless a smaller number is indicated in parentheses

Crow Neck		Hampton Harbor		Bailey's Mistake		Hampton Beach	
½ (L + W) mm.	Pore-pairs	½ (L + W) mm.	Pore-pairs	½ (L + W) mm.	Pore-pairs	½ (L + W) mm.	Pore-pairs
13.9	19.6	—	—	—	—	—	—
20.1	27.1	—	—	—	—	20.9	27.6
28.9 (4)	34.4 (4)	—	—	—	—	25.4	33.7
—	—	—	—	—	—	30.2	36.4
35.3	40.4	—	—	—	—	35.2	43.3
40.3	43.9	40.6	46.9	—	—	40.1	46.3
45.4	47.0	45.1	53.3	—	—	45.1	49.5
51.3	51.6	51.2	55.8	51.7	53.9	51.4	55.5
55.3	55.5	55.2	58.8	55.2	56.1	55.5	59.3
60.2	55.0	60.2	66.9	60.2	61.8	—	—
65.1	57.4	—	—	65.3	63.9	—	—
72.0	63.6	—	—	70.4	67.2	—	—
—	—	—	—	75.1	69.1	—	—
—	—	—	—	81.4	74.0	—	—

living in the other localities studied. Also it would appear that the animals from eastern Maine generally grow faster than those from southern New Hampshire.

Discussion

In this study variations among populations of sand dollars (*E. parma*) from different localities have been noted. These variations pertain to relative linear dimensions, test weight, and numbers of pore-pairs in the petals.

The preponderance of relatively longer specimens from Crow Neck, Maine, where they live in what is essentially a reversible tidal river, and the strong tendency for specimens to be wider than long at Hampton Beach, New Hampshire, Bailey's Mistake, Maine, and Scusset Beach, Massachusetts, where they live on surf-swept beaches, suggest that in some way the nature of the water movement where these animals live may affect their shape or bring about a selection such that longer individuals are selected where there are fairly strong currents flowing in one direction for several hours continuously, and wider specimens are selected where there is a mixture of current, often frequently reversing, and with the pounding of breaking waves. The occurrence of populations intermediate in this respect in localities where there is little surf, and what appears to be less current, supports the idea that there is some sort of correlation between the relative linear dimensions of these animals and the nature of the environment in which they live.

That there may be correlations between habitat and relative linear dimensions in other scutellinids is suggested by the literature discussing the status of *Dendraster excentricus* var. *elongatus* H. L. Clark 1935. Clark clearly considers it a variety. Grant and Hertlein (1938) at least tentatively accept Clark's opinion. MacGinitie and MacGinitie (1949) think there are two species which differ in form and habitat requirements. Mortensen (1948) considers this variation not to justify even varietal status. Durham (1955), without mention of the taxonomic question, states (p. 159), "It seems that the excentricity of the apical system and the greater posterior development of the food grooves is correlated with this habit [living on the edge where not too strongly affected by wave action]. The non-excentric species [specimens?] probably lie flat on the sea floor."

Thus, there appears to be a somewhat similar problem among populations of *Dendraster* on the Pacific Coast. Because the varietal name *"elongatus"* applied to the populations living in quiet water suggests that the animals are longer than those typical for the species that live on the surf-swept beaches, it would appear that the correlation between relative linear dimensions and water movement in the habitat is the same in *Dendraster* as in *Echinarachnius*. There is, however, a question concerning what Clark (1935) considers length. He describes the holotype as being 41.5 mm. long and 40 mm. wide, but the specimen the MacGinities (1949) illustrate (Figure 100, page 238) is distinctly wider than long. Thus, the question arises as to whether this discrepancy results from differences in terminology—*i.e.*, what is meant by "length"—or from real differences between Clark's and the MacGinities' concept of the variety. To settle this, Clark's holotype (M.C.Z., No. 6040, not 3343 as Clark says) has been examined and measured. It is in fact longer than wide (41.2 mm. long × 40.5 mm. wide by my measurement) as are the two paratypes (M.C.Z., No. 6041). Thus, one is tempted to suspect that Clark (1935) and the MacGinities (1949) had rather different ideas concerning the characteristics of the variety, and careful reading of Clark's (1935) description and the MacGinities' (1949) discussion tends to substantiate this, not only in terms of shape but also in habitat. Even so it would appear that there is need for careful quantitative work aimed at determining the nature of any causal relationship that may exist between linear dimensions and habitat.

As shown in Table III and Figure 3, there appear to be rather distinct differences in the relationships between test weight and mean diameter. The populations from Crow Neck, Maine, and Hampton Beach, New Hampshire, appear to be at the extremes of the four collections examined, with those from Crow Neck being relatively the heaviest and those from Hampton Beach being the lightest. Again the populations from Bailey's Mistake, Maine, and Hampton Harbor, New Hampshire, are intermediate, with those from Bailey's Mistake appearing to be slightly the heavier, but because of the small amount of overlap in size-range between these two populations, this is a questionable difference. It is possible that whatever factors may be responsible for the differences in relative linear dimensions may also be operating here in respect to the relationship between test-weight and mean diameter. However, it should be recalled that Raup (1958) found the tests of the Pacific sand dollar, *Dendraster excentricus*, to be heavier in relation to diameter in populations from cold water than those from warmer water and that the available data appear to indicate that the mean annual water temperatures for the Maine localities are probably some 3° F. lower than those for the New Hampshire localities. That there may be other factors involved can hardly be doubted. H. L. Clark (1948) in an attempt to find a possible causal basis for explaining differences in numbers of coronal plates in tests of the regular sea urchin, *Strongylocentrotus franciscanus*, of comparable sizes suggests (p. 278) that "unusual features of their habitat, such as excessively strong surf or tidal currents" may affect this relationship. Swan (1962) found that specimens of *S. droebachiensis* living off the Gaspé, from shallower and presumably more turbulent water, had more coronal plates than those from deeper and presumably quieter water. Although I have found no statements in the literature on the subject, Swan in conversation tells me he strongly suspects that the tests of the specimens with relatively more numerous plates are also heavier at comparable sizes. In bivalve mollusks there appears to be a significant amount of evidence (Shih, 1937; Swan, 1952) that, more or less generally, factors that reduce the rate of growth tend to increase shell thickness at comparable sizes. Likewise there is a vast literature (*cf.* Barlow, 1961) indicating that fishes grown under conditions retarding their growth rate through critical stages in their development often have larger numbers of meristic structures. It should, however, always be remembered that the skeletons of the mollusks and the vertebrates differ greatly from each other and from the skeletons of echinoderms. Thus, although similarities may appear great, caution should be used when speculating concerning what might happen in one group on the basis of what has been observed in another. That oxygen content of the water could affect shell or test thickness through its effect on ease of precipitation of $CaCO_3$ should be considered. Finally, the possibility of the selection of different genetic types by different sets of environmental factors cannot be ruled out. Under some conditions it is possible that heavier tests would have distinct survival value whereas under others lighter tests might be advantageous. Nichols' (1962) study of differences between populations of heart urchins (*Echinocardium cordatum* Pennant) should be instructive to anyone trying to determine whether morphological differences are selected or induced by environmental differences.

The findings concerning the numbers of pore-pairs in the petals of specimens from the four populations studied in this respect are puzzling in view of Durham's (1955) idea that the older specimens of comparable size would have more plates in the petals and in view of the aforementioned idea that the older specimens would have heavier tests. The fact that the specimens from Crow Neck are heaviest would indicate that they are slow growing, and their having the smallest numbers of pore-pairs in the petals would indicate that they are growing rapidly. Thus, it appears that until studies have been made in which the ages or growth-rates characteristic of populations are definitely determined, test weight and numbers of pore-pairs in the petals must be considered as independent of each other and neither should be considered as having a proven relationship to growth rate.

SUMMARY

1. The widespread occurrence of skeletal variation in echinoids is indicated.

2. Variation in respect to several characteristics of the test has been studied in the sand dollar *Echinarachnius parma* (Lamarck) as it occurs in a number of New England localities.

3. Evidence is presented that indicates a tendency for these animals to produce tests longer than wide when living in flowing water and wider than long when living on surf-swept beaches.

4. In this species, as Raup (1958) reported earlier for *Dendraster* on our west coast, tests tend to be heavier at comparable sizes in populations living in colder water than those of warmer localities.

5. Populations vary from one another in numbers of pore-pairs in the petaloid areas of the ambulacra of individuals at comparable sizes, but no consistent correlations with environmental factors have been detected.

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SYMBIOSIS OF HYDRA AND ALGAE. I. EFFECTS OF SOME ENVIRONMENTAL CATIONS ON GROWTH OF SYMBIOTIC AND APOSYMBIOTIC HYDRA

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Unicellular algae inhabit a variety of aquatic invertebrates. Their primary function as symbionts is in most cases not clearly understood (see review of Droop, 1963). Interest in this problem has led us to an experimental analysis of the association of algae and hydra. Green hydra are particularly suited to an investigation of symbiosis involving a metazoan because (1) methods for mass culture of these hydroids provide a practically unlimited supply of animals of similar genetic, developmental, and nutritional histories, cultured in a fluid of known ionic composition. (2) Aposymbiotic (= algae-free) control hydra are easily obtained (Whitney, 1907) and cultured, and (3) the specific growth rate constant, k (*cf.* Loomis, 1954), provides a quantitative measure of the effect of various factors, such as ionic composition of the medium, or the presence of symbiotic algae, on growth of the host.

In preparation for studies on the effect of symbiotic algae on growth of hydra, it was necessary first to attempt to control environmental variables affecting growth. Since the most critical of these for laboratory-grown hydroids appears to be the ionic composition of the culture medium (Loomis, 1954; Loomis and Lenhoff, 1956; Ham, Fitzgerald and Eakin, 1956; Lenhoff and Bovaird, 1959, 1960; Fulton, 1962), the experiments described here were undertaken to determine the effect of some environmental cations on growth of symbiotic and aposymbiotic hydra.

MATERIALS AND METHODS

Chlorohydra viridissima, obtained from the Carolina Biological Supply Co., Burlington, N. C., and designated by us "Carolina Strain 1960," was used in all experiments.

The general morphology of green hydra and associated algae is described by Goetsch (1924), Haffner (1925), and Brien and Reniers-Decoen (1950). Present knowledge of symbiotic algae is summarized by Droop (1963).

Carolina Strain 1960, with an average resting length of about 5 mm., is small compared to other green hydra. Usually 15–25 unicellular green algae, each 3–6 microns in diameter, are situated basally within most of the host's gastrodermal cells. Electron micrographs of *C. viridissima* (Wood, 1959) show the intracellular location of the algae.

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The culture medium ("M" solution) for this species of hydra consisted of 10^{-3} *M* $CaCl_2$, 10^{-3} *M* $NaHCO_3$, 10^{-4} *M* $MgCl_2$, 10^{-4} *M* KCl, and 10^{-3} *M* tris-(hydroxymethyl)-aminomethane buffer (Sigma "121"), pH 7.6, in water deionized by passing distilled water through organic removal and ion exchange resin columns (Barnstead Black and Red Cap Cartridges). The experimental rationale for the selection of this medium is given in the Results section (Fig. 2). Penicillin G (sodium, U.S.P., 50 mg./L.) was occasionally added to stock cultures to retard growth of contaminating microorganisms, but was omitted from cultures during experiments.

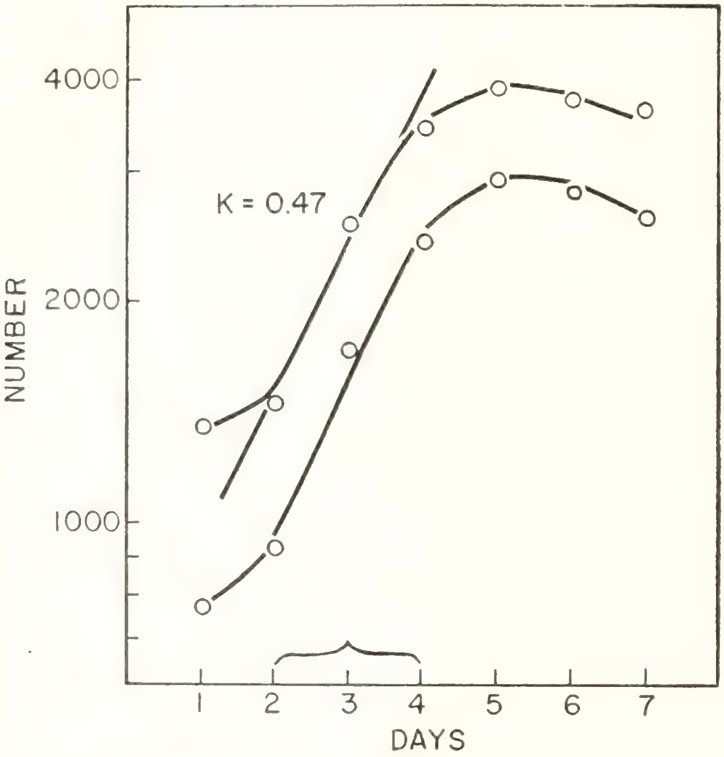

FIGURE 1. Growth curves for a stock culture of *C. viridissima* (green) obtained by counting hydranths (upper curve) or individual hydra (lower curve). Sampling period during logarithmic growth indicated by bracket.

Aposymbiotic control animals (referred to hereafter as albinos) were obtained by growing green individuals for eight days in culture solution containing 0.068 *M* glycerine (Whitney, 1907, 1908). Following this treatment, the absence of algae was confirmed by microscopic examination of macerated tissues and of 2-micron sections of paraffin-embedded whole animals. Aposymbiosis was permanent. Occasionally, algae reappeared in some individuals soon after glycerine treatment, apparently as a result of incomplete removal of algae. Under conditions defined elsewhere in this paper, albinos grew normally when returned to glycerine-free culture solution, and have continued to do so through three years

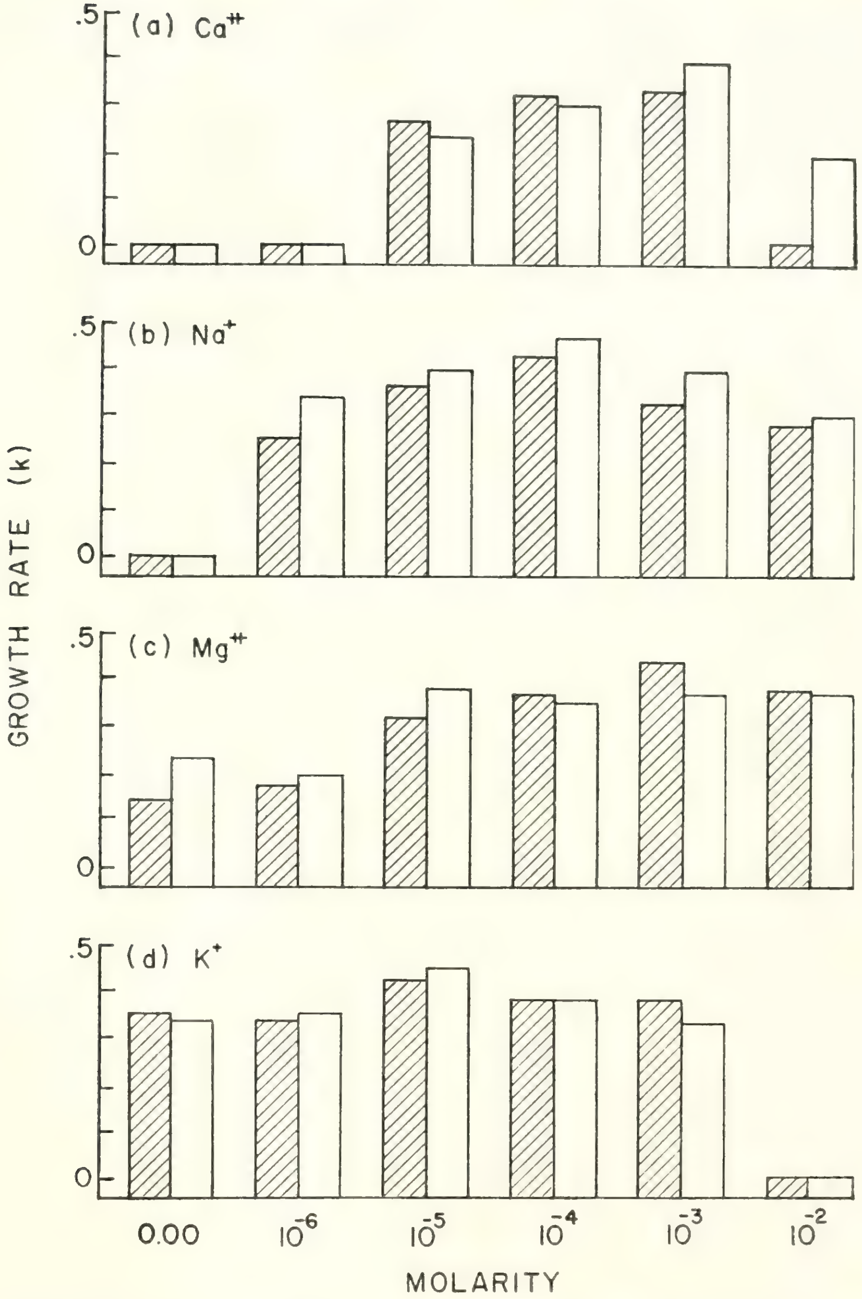

FIGURE 2. Histogram showing the growth rates of green (diagonal lined bars) and albino (open bars) *C. viridissima* in "M" solution in which the concentration of each of four cations is varied in turn while others held constant.

of subculture in our laboratory. Since the approximate life span of a *C. viridissima* cell is 2–3 weeks (Brien and Reniers-Decoen, 1949, 1950; Burnett and Garofalo, 1960), any undesirable effects of glycerine treatment would presumably be diluted or absent after a few months of subculture.

Stocks of hydra were cultured in Pyrex trays (33 cm. × 22 cm. × 4.5 cm.) and were fed daily on freshly hatched *Artemia* nauplii (*cf.* Loomis and Lenhoff, 1956). The hydra were kept at ambient laboratory temperatures (21–24° C.) and illumination; additional illumination was provided continuously from a 40-watt fluorescent light (Sylvania-Cool White) kept 15 cm. from the center of a stack of 2–4 trays. Stock cultures attaining a maximum of 4000 hydra/1500 ml. of culture solution were thinned and cleaned weekly by pooling the animals, washing them in deionized water and finally re-distributing 600–800 hydra (1200–1500 hydranths) to a tray containing 1500 ml. of clean culture solution. Throughout the course of these experiments, *C. viridissima* strain 1960 reproduced exclusively by asexual budding.

Hydra used in experiments were sampled from asexually-reproducing stock cultures during the logarithmic growth phase as illustrated in Figure 1. The maximum specific growth rate constant (k_{max}) for the stock population compares favorably with those values obtained for the growth rates of small experimental cultures (compare Fig. 1, Table II). Each sample consisted of duplicate groups of five hydra, each with a bud in an early stage of development, that were starved one day prior to the experiment. These animals can be defined as five "uniform" hydra (Lenhoff and Bovaird, 1961) or ten hydranths. Strict adherence to these sampling criteria was essential for reproducible experimental results.

Growth was measured by the procedure of Loomis (1954). Five uniform hydra were placed in 30 ml. of culture solution in a 10-cm. (diameter) Petri dish placed 10 cm. from a single 40-watt fluorescent light (Sylvania-Cool White).

TABLE I

Growth of green and albino C. viridissima *in unmodified and modified culture solution*

Culture solution	Number of hydranths on day						Growth rate k
	1	2	3	4	5	6	
A. "M" solution							
Green	10	15	25	32	50	91	0.433
Albino	10	16	26	38	53	87	0.433
B. "M" solution with 10^{-3} *M* NaCl instead of $NaHCO_3$							
Green	10	17	28	43	75	114	0.462
Albino	10	15	28	46	69	118	0.477
C. "M" solution plus 0.05 g./L. penicillin							
Green	10	15	23	40	60	105	0.477
Albino	10	15	23	38	60	100	0.462
D. "M" solution plus 0.068 *M* glycerine							
Green	10	15	19	30	38	64	0.355
Albino	10	14	18	27	43	57	0.346

TABLE II

Summary of growth rates of green and albino (*) C. viridissima *measured prior to July, 1962. Culture conditions defined in text*

Group	No. of replicates	Mean k	Range
1	2	0.46	0.00
2	6	0.45	0.03
3	2, 2*	0.45	0.06
4	2, 2*	0.43	0.05
5	3	0.46	0.11
6	2	0.49	0.00
7	2	0.55	0.04
8	2	0.45	0.08
9	2	0.43	0.05
10	2	0.42	0.02
11	3	0.43	0.04
12	2*	0.45	0.04
13	2	0.42	0.01
14	2	0.45	0.05
15	2*	0.39	0.00
16	2	0.31	0.06
17	2	0.37	0.01
18	1, 2*	0.39	0.04
19	3	0.32	0.02
20	2	0.32	0.02
21	2*	0.36	0.01
Mean values			
entire group	(n = 54)	0.42 ± 0.06	0.29
green only	(n = 42)	0.42 ± 0.06	0.29
albino only	(n = 12)	0.41 ± 0.03	0.12

The number of hydranths was counted daily and then a dense suspension of *Artemia* nauplii was introduced to each hydranth in the dish with a glass pipette. Hydranths which happened not to catch larvae within the first few minutes were given *Artemia* with watchmaker's forceps. The culture medium was renewed one hour after feeding and again six hours later after the normal process of regurgitation was completed. This procedure was followed for 5–7 days. A semilogarithmic plot of the number of hydranths present on successive days (*cf.* Table Ia) yielded a series of points. From a straight line fitted to these points, k, the growth rate constant, was calculated using the standard equations for logarithmic growth. These can be expressed finally as $k = \ln 2/T$, where T is the doubling time in days, estimated to the nearest tenth of a day (*cf.* Loomis, 1954).

RESULTS

Environmental cations required for growth

Figure 2 shows the results of a series of growth experiments in which the concentration of one environmental cation was varied while that of the others was kept constant as in "M" solution. The effect of the varied cation on growth of green and albino *C. viridissima* was determined from the value for *k*. Growth at or below a rate characteristic of starved hydra was assigned a *k* value of 0.00.

C. viridissima required at least 10^{-5} *M* calcium (Fig. 2a) and 10^{-6} *M* sodium (Fig. 2b) ions for growth. In the absence of these ions the animals did not grow, failed to feed, remained contracted and were prone to disintegration within a few days.

In the absence of magnesium ions, budding rates were usually low (Fig. 2c), approaching those of starved animals, but occasionally maximum growth was observed. Addition of at least 10^{-5} *M* magnesium ions always enhanced growth rates.

Although growth occurred without potassium (Fig. 2d) its presence in the medium markedly enhanced the general appearance of the polyps and the reproducibility of growth curves. Average length of tentacles increased about threefold when potassium was present.

High concentration of calcium and potassium inhibited growth (Fig. 2a, d), while similar high concentrations of sodium or magnesium were without effect (Fig. 2b, c).

The general growth response of green and albino *C. viridissima* to all ions tested was nearly the same with two exceptions: (1) albinos appeared more sensitive to lack of environmental sodium, disintegrating several days earlier than green hydra, and (2) green hydra produced significantly fewer buds than did albinos at high calcium concentrations.

Anions and other factors

Growth increased slightly but not significantly when NaCl was substituted for $NaHCO_3$ (Table Ib), but $HCO_3^=$ was retained in all subsequent experiments to insure an ample external carbon dioxide pool for the algae.

When penicillin was added to stock cultures (Table Ic), the growth rate increased slightly. Glycerine (0.068 *M*) decreased the growth rate considerably (Table Id), and was used only to eliminate symbiotic algae.

Growth rate under standard conditions

Table II summarizes the growth rates of *C. viridissima* measured in our laboratory prior to July, 1962. Standard conditions are defined as growth in laboratory culture solution, pH 7.6, 21–24° C., continuous illumination (see Methods), daily feeding on freshly hatched live *Artemia* nauplii, twice-daily changes of the culture solution, constant volume of culture solution per vessel and initiation of growth experiments with uniformly developed hydra of known nutritional history sampled during the logarithmic growth phase of stock populations. The 54 measurements include data for 42 groups of green and 12 of albino *C. viridissima*. Under these conditions, albinos grow at about the same logarithmic rate as green individuals.

Using an analysis of hydroid growth measurements as set forth by Fulton (1962), the standard deviation of the mean growth rate of the whole group (54 measurements) is ±0.06; the range, 0.29. For each individual set of experiments (21 measurements) the standard deviation of the mean is ±0.03 (calculated from ranges), the range, 0.11. Thus, the variation encountered from one group of experiments to another is about twice that encountered among replicates within any single experiment, indicating that conditions within each set of experiments were relatively constant.

In 20 of 21 experiments, the growth rate of replicates differed from each other by 0.08 or less. In one experiment a difference of 0.11 was observed. From these data we may estimate that 95% of the time a growth rate difference of 0.08 or more between cultures is significant.

DISCUSSION

The results demonstrate that in a solution containing calcium, sodium, magnesium, potassium, chloride, and bicarbonate in the concentrations described, growth of *C. viridissima,* Carolina strain 1960, is exponential with a mean k of 0.42 ± 0.06. This represents a doubling time of 1.45–1.90 days. This is the fastest mean growth rate thus far encountered among laboratory-grown hydroids for which there are comparable data (see Table III) and in part reflects the smaller size of *C. viridissima.* The observation that the mean growth rates of green and albino *C. viridissima* are nearly identical *under the conditions described* suggests that the algae are not essential for growth at k_{max}. This observation is dealt with in the next paper in this series.

TABLE III

Comparison of ionic requirements for growth of several hydroids in laboratory culture. Table modified from Fulton (1962)

	*H. littoralis**	*C. lacustris*	*C. viridissima*
Growth curve	exponential	exponential	exponential
k	0.37	0.21	0.42
Cations required	Ca^{++} (10^{-5} M) Na^{+} (10^{-6} M)**	Ca^{++} (10^{-3} M) Na^{+} (10^{-3} M) K^{+} (10^{-3} M)	Ca^{++} (10^{-5} M) Na^{++} (10^{-6} M)
Cations which enhance growth but are not required	K^{+}***	Mg^{++}	K^{+}, Mg^{++}

* Data of Loomis (1954).
** Data of Lenhoff and Bovaird (1960).
*** Data of Lenhoff (unpub.).

Using data of Loomis (1954) and Fulton (1962) some growth features of *Hydra littoralis* (a stream-dwelling non-symbiotic species, about 3–4 times larger than *C. viridissima*) and *Cordylophora lacustris* (a colonial hydroid typically found in fresh to brackish situations) are compared with those of *C. viridissima* in Table III. All have been grown in the laboratory under controlled conditions. All three hydroids require calcium ions in the environment for growth, although the minimal required concentrations vary with species. In the absence of calcium, the animals disintegrate very quickly, reflecting the fundamental role of this ion in maintaining cell and tissue integrity. At low levels of calcium, other factors which may adversely affect growth are (1) loss of calcium-dependent contractility necessary for positioning body and tentacles in prey capture and feeding; (2) failure of nematocysts to discharge even when contacted by live *Artemia* nauplii; (3) inability to carry out the feeding reflex for which environmental calcium is required (Lenhoff and Bovaird, 1959). The animals are thus effectively prevented from feeding and obtaining additional calcium from food.

C. viridissima also shares with *Hydra littoralis* and *Cordylophora* an absolute requirement for sodium in the external environment. Only trace amounts of sodium appear necessary for near-optimum growth of the fresh-water hydrids (*H. littoralis* and *C. viridissima*), while *Cordylophora*, as expected from its habitat, tolerates a relatively higher environmental sodium concentration.

Environmental potassium ions are not an absolute requirement for growth of *C. viridissima* or *Hydra littoralis,* but when present, even at low concentration (10^{-6} *M*) enhance the general appearance of polyps and the level of reproducibility of growth rates. The dramatic increase in length of tentacles observed when potassium is introduced into the medium may reflect the role of this cation in nerve and muscle irritability. Since tentacle length:body length ratio is often used as a taxonomic character in identification of hydras (Hyman, 1929), standardized culture media may help eliminate uncertainty in this parameter.

Although environmental magnesium definitely enhances growth of *C. viridissima* (*cf.* Muscatine, 1961), it is uncertain as to whether or not it is absolutely required since maximum growth was occasionally observed in the absence of this cation. Magnesium also appears to be "less critical" for *Cordylophora* (Fulton, 1962). Acquisition of magnesium from food may offset a deficiency in the medium and thus interfere with the reproducibility of the effects of absence of environmental magnesium on growth. A magnesium requirement for *C. viridissima* is apparently not primarily related to a requirement by the algae since the growth rate of albinos is also lower in the absence of magnesium.

Inhibition of growth of *C. viridissima* at high concentrations (10^{-2} *M*) of calcium and potassium was probably not an osmotic effect, but more likely related to competition with other ions since similar osmolar concentrations of sodium and magnesium did not inhibit growth. Sodium-potassium competition is a well-known phenomenon, and has been demonstrated in hydroids by Fulton (1962) in growth experiments using *Cordylophora.*

There is evidence that symbiotic algae may affect mechanisms of salt and water balance of the host. For example, in these studies albino *C. viridissima* disintegrated several days sooner than green *C. viridissima* in the absence of sodium. The same effect was observed in a medium also lacking in magnesium (Lenhoff and Bovaird, 1960). On the other hand, albinos were less sensitive to high calcium compared to green individuals. Karakashian (1963) noted that algae-free *Paramecium bursaria* crenated and died within a few hours after inoculation into an inorganic salt medium in which normal green individuals could be maintained. Hood (1927) observed that specimens of *Frontonia leucas,* another ciliate harboring symbiotic algae, lived indefinitely in a 1% dextrose solution changed daily, and for several days when adapted to 3% dextrose. In contrast, algae-free individuals averaged two days' survival in 1% dextrose and died within a few hours in more concentrated solutions. However, in distilled water green specimens disintegrated immediately while those without symbionts survived for several hours.

Part of this investigation was carried out at the Laboratories of Biochemistry, Howard Hughes Medical Institute, Miami, Florida, during the tenure of a Postdoctoral Fellowship from the Division of General Medical Sciences, United States Public Health Service (9653) to Leonard Muscatine, and an Investigator Award of the Howard Hughes Medical Institute to Howard M. Lenhoff. We thank Mr. John A. Bovaird, Mr. Alfredo Lopez, and Mr. Enrique Nagid for technical assistance.

Summary

1. Under controlled conditions in the laboratory, with daily feeding, *Chlorohydra viridissima* grew exponentially in a solution containing calcium, sodium, magnesium, potassium, chloride and bicarbonate ions. The optimum concentration of cations was ascertained and the effect of their deficiencies noted. The results are compared with data for *Hydra littoralis* and *Cordylophora lacustris.*

2. Calcium and sodium ions were required for growth. Magnesium and potassium enhanced growth and reproducibility of growth rates. Bicarbonate was not essential.

3. Both symbiotic and aposymbiotic *C. viridissima* grew at nearly identical rates, doubling in number every 1.45–1.90 days. In the absence of environmental sodium aposymbiotic hydra disintegrated several days sooner than normal green individuals, while growth of the latter was inhibited by high concentrations of calcium in the environment.

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EFFECTS OF X-IRRADIATION OF MICE EXPOSED IN UTERO DURING DIFFERENT STAGES OF EMBRYOLOGICAL DEVELOPMENT ON DURATION OF MATURE LIFE[1]

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In an earlier paper (Nash and Gowen, 1962) we have shown that postnatal growth, as measured by weight changes to 75 days, following x-ray irradiation *in utero,* was dependent on both the levels of irradiation and the embryological ages at the time of irradiation. The embryological ages in order of increasing sensitivity were 6½, 17½, 14½ and 10½ days. Growth was measured for the periods from mating to birth, and to 12, 26, 40, 60 and 75 days postnatal ages. Body weight response was found to be noticeably dependent upon the age at which observations were recorded. Treatments that produced significantly lowered body weights did not usually show maximum effects until 40 days after birth. Growth changes, expressed as continuing reductions of the body weights with days post-parturition, were most obvious at 75 days of age. The reductions were greatest for the 320 r-treated mice, the lowering of the growth rates being dose-dependent.

The data on which these observations were made have one common property; the mice were alive at the time when the observations were initiated. This results in the population under observation being biased toward greater viability. This limitation is intrinsic at whatever level of development the data on the irradiation effects are taken. To, in part, reduce this difficulty in evaluating the irradiation effects on the animal's subsequent well-being, several other characteristics, such as anatomical changes in young born dead, lifetime fecundity and fertility and various parts of the lifespan, have been utilized for measuring as yet unrecognized physiological changes in those exposed to irradiation. This paper deals with those changes, which may be immediate or long delayed, which affect lifespan after the beginning of the reproductive period.

Lifespan is a unique instrument in that it integrates the effects of external as well as internal agents influencing life processes. It covers both the short-term and long-term effects as well as the constitutional characteristics of those exposed. It has more variation, both innate and accidental, than other measures so differences between agents believed to affect life become harder to demonstrate. Yet it is in this characteristic most of us are interested.

Irradiation of the mammalian embryo exposes rapidly dividing cells during the intense process of organ formation and differentiation. It is a time when gene and chromosome changes would most likely result from single acute irradiation. Mitotic changes in single cells should grow to islands of tissue having functions different from those of the rest of the body complex or organ. The changes in the compatibility of the functioning units can influence those of the body as a whole and be expressed in changes in life's duration (Gowen, 1962).

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Materials and Methods

The embryos irradiated were obtained exclusively from first pregnancies in matings within and among three of our long-inbred strains of mice. After mating, the mothers marked for subsequent irradiation were examined daily for the presence of a vaginal plug. The appearance of this plug was considered the time of fertilization and the start of the events covered by gestation. Embryo ages were calculated from this point. All mice were considered to have mated during the same time at night, with 4 AM as the most likely point for the union of sperm and egg. Irradiations were begun at 4 PM; embryological ages at irradiation were set at $6\frac{1}{2}$, $10\frac{1}{2}$, $14\frac{1}{2}$ and $17\frac{1}{2}$ days. In a later series, irradiations of newborn young were indicated as of $19\frac{1}{2}$ days of age. These mice add breadth to the observations in that they alone are irradiated, whereas both mothers and young are treated at the earlier ages with the possibility that a maternal effect may be added to the direct effect of irradiation on the progeny.

The inbred females irradiated were of strains long under study and differentiation at the Genetics Laboratory of Iowa State University. The major criteria of differentiation have been relative resistance to several diseases of bacterial or virus origin, chemical poisons and radiant energy (Schott, 1932; Gowen and Schott, 1933a, 1933b; Hetzer, 1937; Gowen and Zelle, 1945; Gowen, 1955a, 1955b; Gowen and Stadler, 1956; Stadler and Gowen, 1957a, 1957b, 1957c; Gowen, 1960; Stadler and Gowen, 1961, 1963). The Committee on Mouse Nomenclature has designated the strains as BALB/Gw, K and S. The three strains cover a wide range of susceptibility to radiation. The BALB/Gw (hereafter called Ba) and K strains are relatively susceptible to acute irradiation, whereas the S is relatively resistant.

All progeny were weaned at 30 days of age and males separated from the females. At 75 days of age mice were individually mated to non-irradiated mice of the Z strain. This strain has an entirely different origin than the Ba, K or S. It is noted for regularity, frequency and size of litters. Previously unmated Z males or females were used in these matings. The treated animals were mated to Z mates throughout their lives. To have balanced numbers of mice tested for lifespan and reproductive performance, two males and two females were selected when possible from each treatment group. The after-mating lifespans of these treated animals start at the day of mating and run to the day of death. The full lifespan from birth of any mouse is determined by adding 75 days to the mating-to-death interval. If a litter did not contain two males and two females in each treatment group, an additional litter from another female, raised to 75 days, was used to obtain the additional mice to complete the cell, two each of treated males or females.

When a Z mate died before its treated partner it was replaced within a few days by another virgin Z mouse of an age between two and six months. All matings were retained for the lifetime of the treated mouse.

All matings were observed daily and the following information taken: (a) lifespan of each treated mouse, (b) total number of litters, (c) number of progeny within litters, (d) birth weights of individuals, (e) viability of mice within litters from birth to 21 days, and (f) any external anomalies which the mouse might carry.

Irradiation exposures were chosen to cover the range from none to 320 roentgens, the highest exposure that would allow survival of at least some of the embryos in the embryonic age series. There were five exposures, 0, 20, 80, 160 and 320 r. Five stages in embryological development were treated with the chosen dosages of irradiation. The embryos treated were either males or females of which samples of two each were chosen. The genotypes were divided into nine groups; three represented the pure inbred strains, six their possible hybrids made reciprocally. The design is a factorial in which the treatments are regarded as fixed points. Unfortunately, of the mice treated with 320 r at $6\frac{1}{2}$, with 160 r and 320 r at $10\frac{1}{2}$, none survived for lifespan determinations, and of the 320 r at $14\frac{1}{2}$ embryos only three progeny remained for determining lifespans for this group. In consequence, the design as a whole loses its orthogonality. However, the greater portions of the data may be arranged in different designs to retain the orthogonal principle even though they require separate analyses.

A General Electric Maxitron operated at 250 pkv, 30 ma with 0.25 mm. Cu + 1 mm. Al filtration at a distance of 50 cm. from anode to mid-mouse, dose rate 133 r/minute, furnished the x-rays for the treatments. Dose was measured in air by a rate meter at a position corresponding to that of the mid-mouse. Pregnant mice were exposed to single doses of whole-body irradiation in circular wooden containers $6\frac{1}{2}$ inches in diameter and 1 inch deep. The base of the container was $\frac{1}{4} \times \frac{1}{4}$ inch hardware cloth. The top was covered with two layers of cellophane. Newborn mice of a whole litter were irradiated whole body in small plastic trays and then immediately returned to their mothers. Following custom, all roentgen dose measurements were taken in air at the mid-position the mouse would occupy when irradiated. These dosages do not represent the energies actually reaching different organs of the exposed mouse. They are subject to different adjustments dependent on the particular tissue and absorptions in different body tissues having characteristic atomic densities. If, for instance, the roentgen dose of 250 pkv, 30 ma filtered through 0.25 mm. Cu and 1 mm. Al to an adult female mouse is measured as 1 r, the dose when absorbed in passing through bone and back muscles to the ovaries becomes about 0.85 r at the position of the ovaries. The reader should take these effects into account. They will be discussed at some later date.

The mice throughout were kept in a well ventilated room, in which the environment and management were relatively constant. Compressed food pellets and water were before the mice at all times.

Frequency Distributions of Post-75-day Lifespans as Affected by Sex Treated, Previous Early Irradiation Exposures While in utero or at Birth, Embryological Ages at Treatments, and Genotypes of Strains Treated

Frequency distributions, even though based on rather small numbers, assist in evaluating the effectiveness and mode of action of the different treatment agents on the adult lifespans of those exposed. These distributions, for treated males and for treated females, are shown at the top of Figure 1.

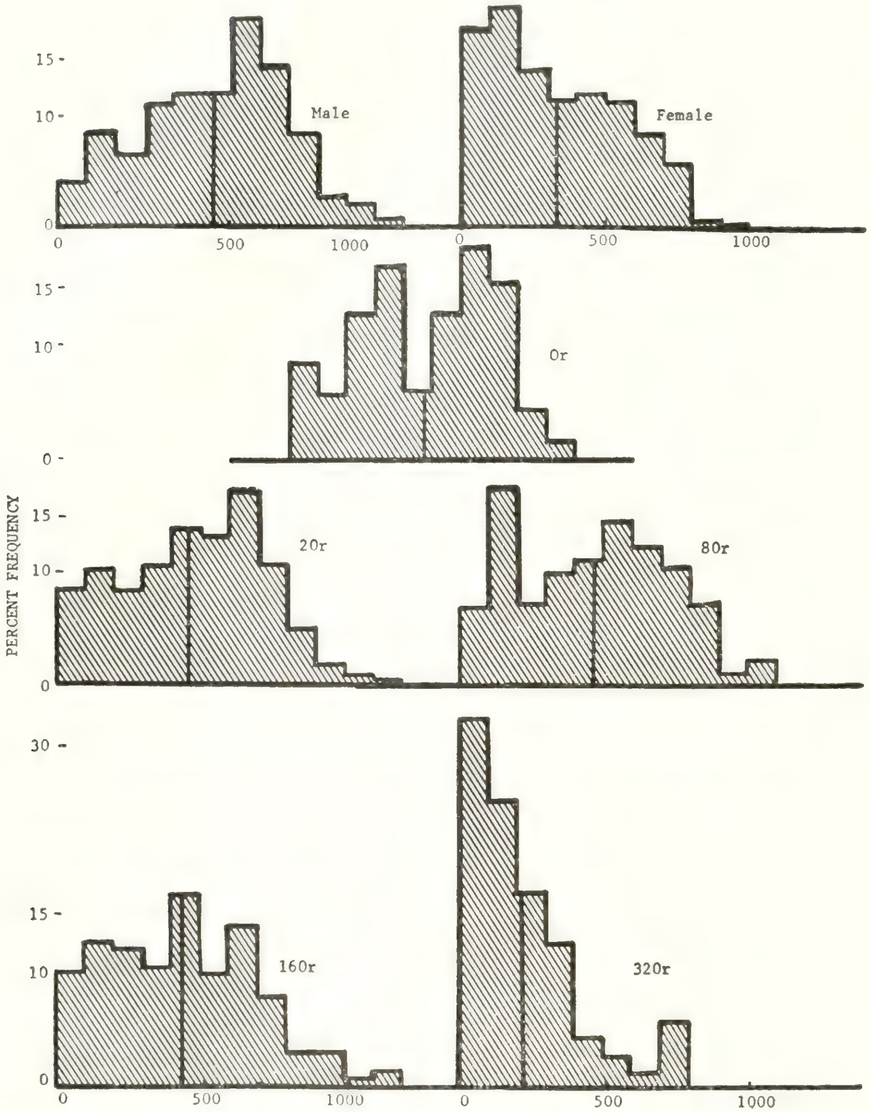

Figure 1. Frequency distributions of adult lifespans of male treated or female treated mice are shown at the top of the chart. The effects of different x-ray dosages 0 r, 20 r, 80 r, 160 r, 320 r are shown in lower part of chart.

The lower three-fourths of Figure 1 gives the frequency distributions for length of life from mating to death of those mice unexposed to x-irradiation, exposed to 20 r, to 80 r, to 160 r or to 320 r at some time from conception to those which are newborn. The viewer should adjust any conclusions derived from these frequencies to the fact that no mice survived to 94 days post-conception in those treatments previously specified.

The distribution of adult male lives shows a longer mean life than that for the females. The forms of the distributions are also different. The male histogram has its modal lifespan at a higher age than that for the mean. The histogram for the female is less symmetrical than that for the males, with the modal value less than that for the treated males. Skewness is positive in contrast to that for the males being negative. The frequency distribution for the females departs significantly from normality.

The five histograms for mice untreated with x-irradiation, 0 r, and those

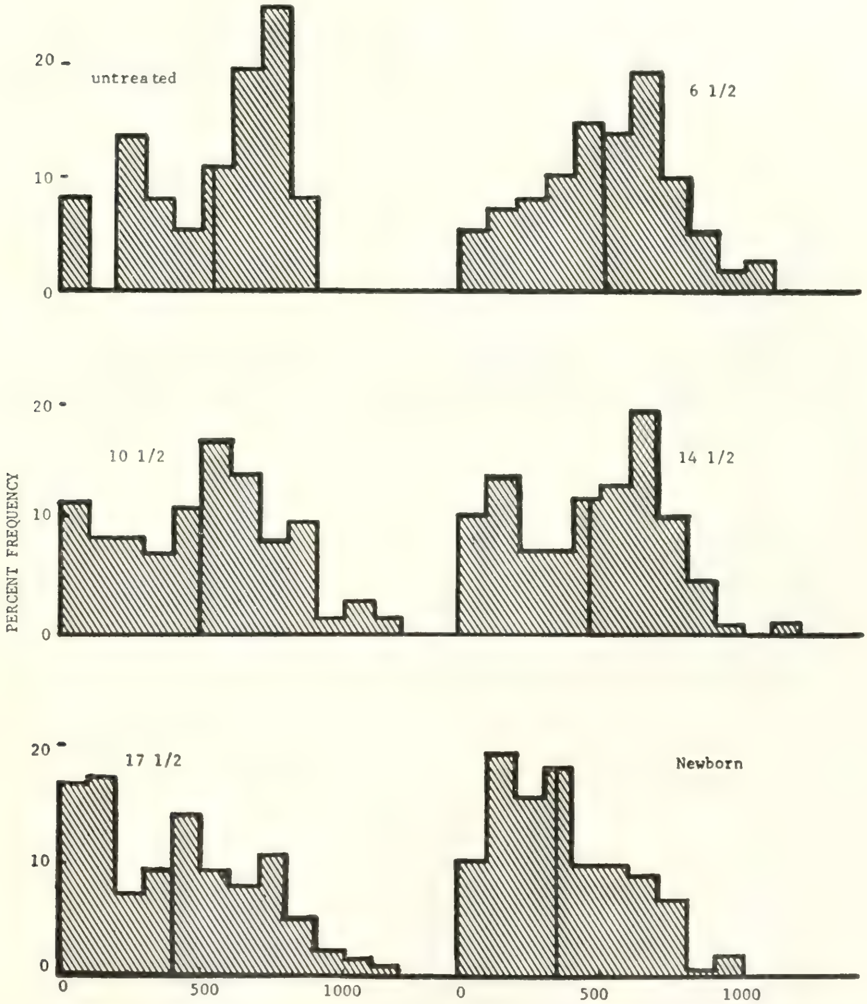

Figure 2. Histograms showing frequencies of adult lifespans of mice exposed to x-irradiation at different periods in embryological development. The charts show the per cent frequencies of those living within each 100-day age group from the date of mating, 75 days of age, to the last death in the populations.

exposed to 20 r, 80 r, 160 r and 320 r are presented in the lower part of Figure 1. These histograms show noticeable differences in form and, in consequence, in their means, modes, variances and skewnesses. The most striking difference is the J-shaped distribution of the 320 r treated mice. Deaths occur earlier and more rapidly than in the other distributions for the lower irradiation dosages. This 320 r histogram presumably would be even more extreme if proper account could be taken of all the classes of mice which were treated with 320 r. Death *in utero* from the 160 r at 10½ days also modified the 160 r histogram in removing these mice from the recorded observations. Later analyses will attempt to evaluate these effects.

Figure 2 shows the distributions of the post-75-day deaths by successive 100-day age intervals. The untreated mice die most frequently at the higher ages. The distribution for the mice exposed *in utero* at 6½ days shows similar but less extreme features. However, the skewness may be partly illusory, for, as indicated, the

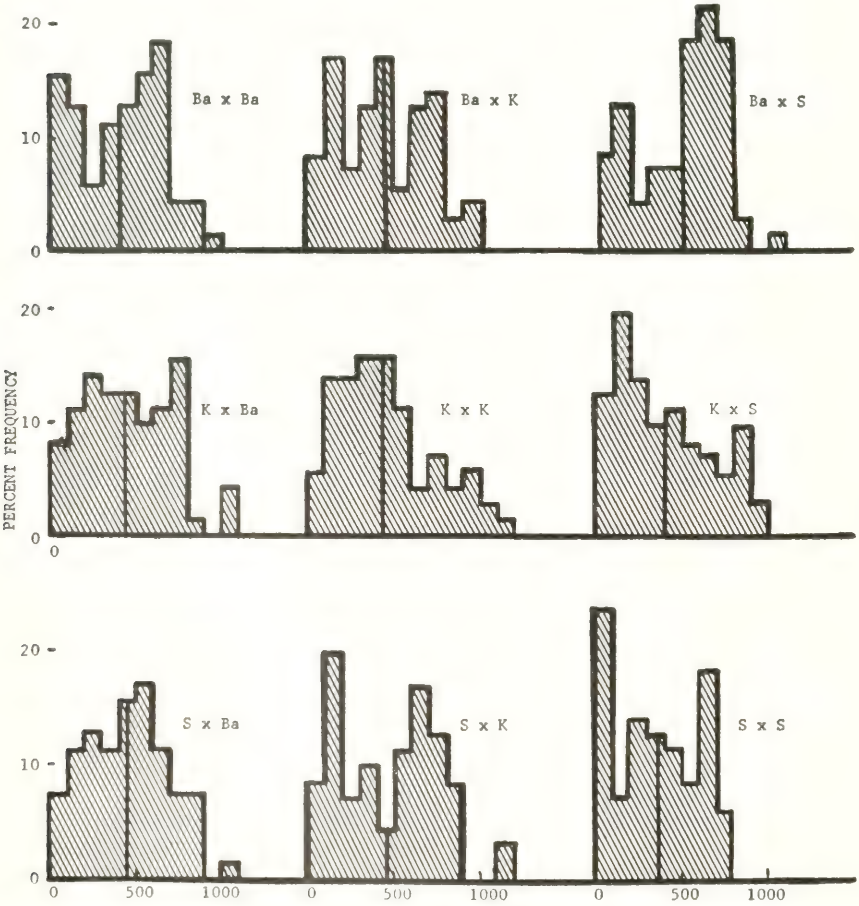

FIGURE 3. Histograms for the three inbred strains and their possible hybrids, showing the percentage frequencies of deaths for each 100-day interval after mating.

distribution does not contain the 320-r irradiated mice. The same considerations apply to the histograms for 10½-day and 14½-day mice. The 17½-day and newborn data are orthogonal since they include all treatments. They are different from the control mice in having their higher frequencies of deaths during the earlier ages. The adult lifespans are positively skewed as contrasted with the negatively skewed characteristics of the un-x-rayed population of mice.

Similar histograms have been drawn for the populations of mice of the 9 different genotypes. The data are also arranged to show the differences between the pure inbred strains and their reciprocal hybrids (Fig. 3). As may be expected the histograms vary widely. The inbreds Ba × Ba and S × S show some tendency to U-shaped distributions although this form is unlikely on both biological and theoretical grounds. The S × S distribution is furthermore not in conformity with our other extensive data, in that this strain ordinarily shows much higher resistance to irradiation, lifespans of 800 days not being uncommon when the irradiation occurs later in life, 46 days from birth, than in the embryonic period. The data sample is also anomalous in having the high frequency of deaths in the 100 days immediately after mating. The ranges in lifespans of the other genotypes are fairly comparable to our earlier data. The histograms show examples of both positive and negative skewness.

The relations between the percentage frequencies of death on the different 100-day age intervals after mating for inbred vs. hybrid genotypes are shown for the combined data at the top of Figure 4. The individual comparisons between the three inbreds and their individual crosses and reciprocals are shown in nine histograms in the lower three-fourths of the chart. The hybrids' average lifespans are longer than the inbreds'. However, some inbreds live as long as any hybrids. The reduction in the average comes in fewer animals living over 700 to 1200 days. There is a tendency toward rectangular distributions of the death frequencies to 800 days from the mating date.

Mature Lifespans Distributed According to Sex, X-ray Exposure, Embryological Age, Strain, Inbred, Hybrid or Reciprocal Crosses

The constants for the frequency distributions (Figures 1 including 4), presented in Table I analyze the characteristics of the different curves and allow an over-all estimation of the frequency curve types in Pearson's systematic treatment of the problem, from the values of β_1 and β_2. Unfortunately, the numbers of individuals in the finer divisions of the populations are frequently smaller than desirable.

Sex effects on survival are noticeable in the means and medians of the mature lifespans. The mean and median and its related function, the mode, establish the curve position on the axis. If the median is the larger, as for the male mice, the curve ranges from nearly symmetrical to negatively skewed, as shown by the g_1 values. The β_2 constant estimates whether the curve is more flat-topped or more peaked, the kurtosis, than the so-called "normal" curve. The significance of the deviations is shown by the t values for g_1 and g_2. The males show greater durations of life after maturity, somewhat more variation in survival time; their frequency distribution is negatively skewed and has significant kurtosis. The female lives in the maturity period are shorter, the median point in their deaths is earlier and the variations are less than those of the males. The distribution of their life-

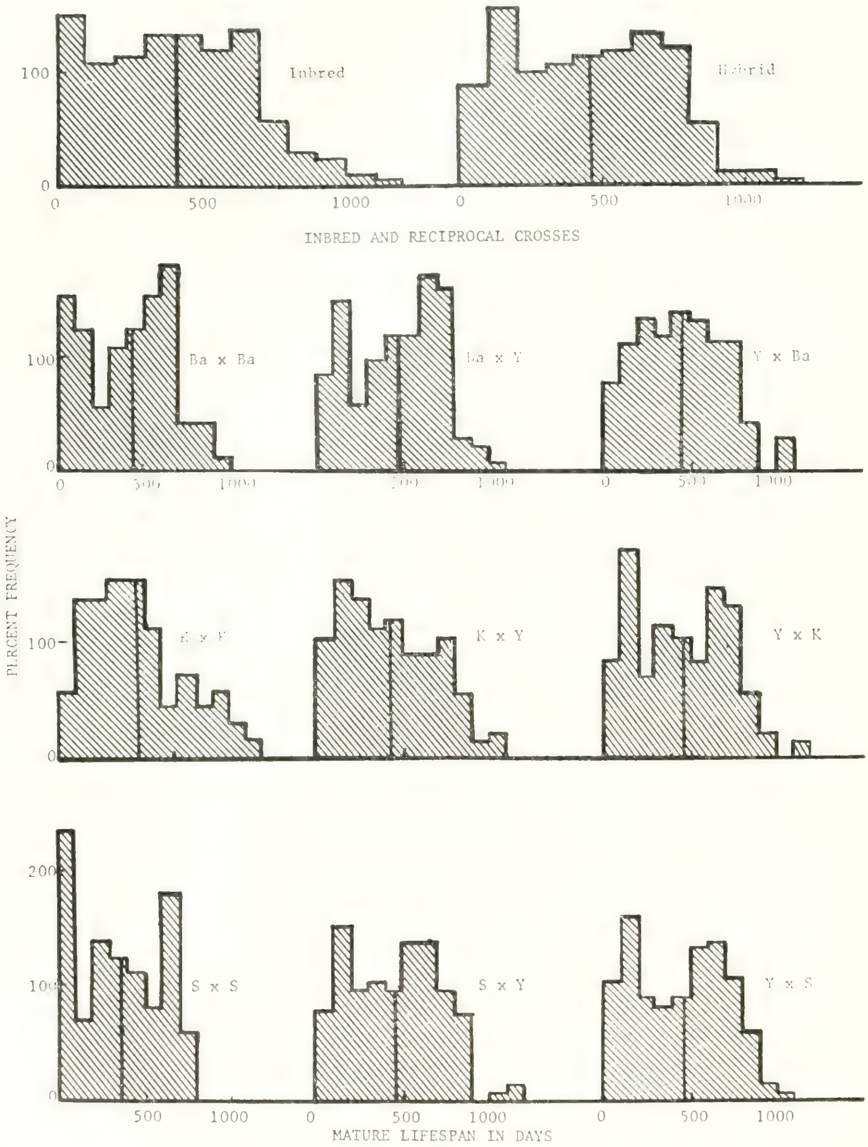

FIGURE 4. Percentage frequencies of deaths plotted on the different 100-day age intervals after mating for combined inbred mice of three strains and their possible hybrids are shown at the top of the chart. The individual inbreds and their possible crosses are plotted in the lower nine histograms, inbreds on the left. Crosses for the males of the same inbred in the middle, *Y* representing the averages of the two crosses. On the right the constant parent is the female inbred of the strain at the left.

TABLE I

Constants for frequency distributions

	Mean	Median	Standard deviation	β_1	β_2	t for g_1	t for g_2
Sexes							
Males	540 ± 14	568	249 ± 8	0.0178	2.405	1.0	2.2 (1)
Females	332 ± 12	299	224 ± 6	0.1627	2.095	3.0 (4)	3.3 (4)
X-ray exposure							
0 r	479 ± 29	505	243 ± 15	0.0416	2.043	0.7	1.7
20 r	474 ± 19	487	250 ± 11	0.0000	2.308	0.0	1.8
80 r	465 ± 19	468	257 ± 10	0.0078	1.991	0.5	2.7 (3)
160 r	438 ± 21	424	257 ± 14	0.0942	2.621	1.5	0.9
320 r	223 ± 22	157	189 ± 19	1.6021	3.946	4.6 (4)	2.0 (1)
Embryological age							
untreated	545 ± 40	601	242 ± 23	0.4270	2.328	1.7	0.8
6½ days	507 ± 23	519	240 ± 14	0.0086	2.512	0.4	1.0
10½ days	493 ± 32	518	276 ± 18	0.0002	2.163	0.5	1.4
14½ days	461 ± 24	493	254 ± 13	0.0046	2.213	0.3	1.7
17½ days	404 ± 23	390	280 ± 12	0.1550	2.112	2.0 (1)	2.2 (1)
Newborn	360 ± 16	337	220 ± 10	0.2391	2.487	2.7 (3)	1.4
Strains							
Ba × Ba	414 ± 29	421	246 ± 14	0.0017	1.922	0.1	1.9
Ba × K	448 ± 31	428	261 ± 15	0.0091	1.933	0.3	1.9
Ba × S	491 ± 29	551	245 ± 15	0.1864	2.131	1.5	1.5
K × Ba	456 ± 31	433	260 ± 18	0.0350	2.304	0.7	1.2
K × K	451 ± 32	404	274 ± 21	0.3833	2.718	2.2 (1)	0.4
K × S	397 ± 31	332	262 ± 15	0.2189	1.983	1.7	1.8
S × Ba	445 ± 28	435	238 ± 15	0.0112	2.211	0.4	1.4
S × K	464 ± 33	514	279 ± 17	0.0159	2.046	0.5	1.7
S × S	359 ± 27	332	231 ± 12	0.0170	1.818	0.5	2.1
Inbreds	407 ± 17	398	254 ± 11	0.1146	2.492	2.1 (1)	1.5
Hybrids	450 ± 12	448	260 ± 6	0.0103	2.035	0.9	4.1 (4)
Reciprocal crosses							
Ba × Y	470 ± 21	499	254 ± 10	0.0250	1.958	0.8	2.5 (2)
Y × Ba	451 ± 21	435	250 ± 12	0.0250	2.290	0.8	1.7
K × Y	427 ± 22	390	263 ± 11	0.1026	2.099	1.6	2.2 (2)
Y × K	456 ± 23	448	270 ± 11	0.0140	2.011	0.6	2.4 (2)
S × Y	454 ± 22	463	260 ± 12	0.0186	2.163	0.7	2.1 (2)
Y × S	444 ± 22	461	258 ± 10	0.0006	1.802	0.1	3.0 (4)

Probability levels (1) = 0.05, (2) = 0.025, (3) = 0.01, (4) = 0.005 or less.

spans is positively skewed, the kurtosis being evident. Both skewness and kurtosis are significantly different from those of the "normal" curve.

The frequency curves for other subdivisions of the data show lowering of the mature lifespans with increasing dosages of the x-ray energy as well as differences between its effects in the different stages in the embryological life when the radiation was received. Inbred mice showed shorter durations of life than hybrids between the strains, and both inbreds and hybrids showed variations dependent on their origins. Negative and positive skewness was observed in the curve forms. Save for one case for mice treated with 320 r, the kurtosis was uniformly negative, reaching significance in 11 out of the 30 distributions.

The differences exerted by the different variables show that analyses of the effects on the mature lives of the preadolescent mice must be analyzed separately for within sex, dosages and embryological ages of treatment.

INFLUENCE OF SEX, X-RAY DOSAGES AND EMBRYOLOGICAL AGES WHEN THE IRRADIATIONS WERE RECEIVED ON MATURE LIFESPAN

Table II presents means and standard deviations for the lengths of the later lifespans distributed for embryological age at irradiation, dose of x-ray exposure and sex of the parent treated. The mean day survival is followed by its standard deviation. The standard deviations of the distributions of the raw data follow.

In every instance the after-mating lifespans of the males are noticeably longer than those of the females whatever may have been the time at which the developing embryos were irradiated, or the amount of irradiation to which they were exposed. This major factor in these lifespan differences, in consequence, is not attributable to the irradiations or the embryological ages at which irradiation occurred. They seem to turn on innate biological differences in the risk of death to the parent in carrying on reproduction. This risk is much greater to the females than to the males. The results support our earlier work in showing that pregnancies spaced at short intervals result in large reductions in the lifespans of the females, whereas the lifespans of the males are nearly unaffected (Gowen, 1960).

Irradiations with 20 and 80 r occurring at the different *in utero* periods showed little effect on the difference in life of the sexes, the females for the same treatments being about 0.6 those of the similarly treated males. A lengthening of the female lifespan occurred when the female was treated with 80 r as a newborn.

MATING-TO-DEATH SURVIVAL FOR MICE IRRADIATED IN UTERO IN RELATION TO DOSE RECEIVED

Figure 5 plots the mean after-mating lifespans of male and female mice distributed within the embryological ages at which the irradiations were given and by the dosage of irradiation they received. The curves for the intrauterine irradiated mice were similar in form. Sex differences in the lengths of the lifespans were marked. Doses to and including 160 r of acute irradiation caused a 72-day loss to males and a 119-day loss to females in their average durations of life, 11% and 27%, respectively, of their expected survivals after mating. The declines in the adult lifespans were irregular but in general followed trends which approach linearity. The reductions in life duration occurred even though the animals had 75 days in which to recover before measurements of the irradiation effects were begun. The groups were selected groups which had eliminated the rapidly fatal irradiation effects.

After the 160-roentgen dosages the effects on later life survival became more severe. Lifespans for mice exposed to 320 r intrauterine were reduced to 43% and 30% of those enjoyed by untreated male and female mice, respectively.

Figure 5 separates the effect of irradiations at different periods in the uterine development. The durations of adult life of males exposed at 6½, 10½, 14½ and 17½

TABLE II

Mean days and standard errors mating to death

Embryological age at irradiation	Irradiation dose	Sex treated	Mean	Standard deviation
Untreated	0 r	Male (M)	646 ± 49	208
		Female (F)	443 ± 57	242
6½ days	20 r	M	597 ± 55	233
		F	373 ± 56	237
	80 r	M	637 ± 55	234
		F	383 ± 54	228
	160 r	M	576 ± 58	246
		F	472 ± 34	143
10½	20 r	M	616 ± 47	201
		F	368 ± 63	268
	80 r	M	586 ± 70	297
		F	401 ± 63	269
14½ days	20 r	M	620 ± 33	141
		F	414 ± 57	244
	80 r	M	590 ± 48	210
		F	313 ± 49	206
	160 r	M	596 ± 60	256
		F	234 ± 43	184
	320 r	M	254	
		F	35	
17½ days	20 r	M	668 ± 55	232
		F	359 ± 49	210
	80 r	M	591 ± 63	266
		F	363 ± 50	212
	160 r	M	551 ± 65	275
		F	267 ± 48	202
	320 r	M	284 ± 61	259
		F	150 ± 39	165
Newborn	20 r	M	447 ± 41	174
		F	275 ± 51	216
	80 r	M	440 ± 50	211
		F	350 ± 50	213
	160 r	M	466 ± 62	263
		F	335 ± 49	209
	320 r	M	289 ± 35	151
		F	169 ± 31	133
Newborn	0 r	M	518 ± 52	220
		F	309 ± 46	194

days of uterine development are quite comparable. The lifespans for males treated as newborns are shorter than expected for the 0, 20, 80 and 160 r treatments. Females irradiated at 14½ days uterine development with 160 and 320 r had their durations of life noticeably reduced. Newborn females either untreated or exposed to 20 r showed noticeably shorter lifespans than expected. No explanation is known which will account for this difference. The newborn group was selected for study some time after the other treatment groups, but the mice in the group were, so far as known, similar to the others previously treated with irradiation when they were chosen.

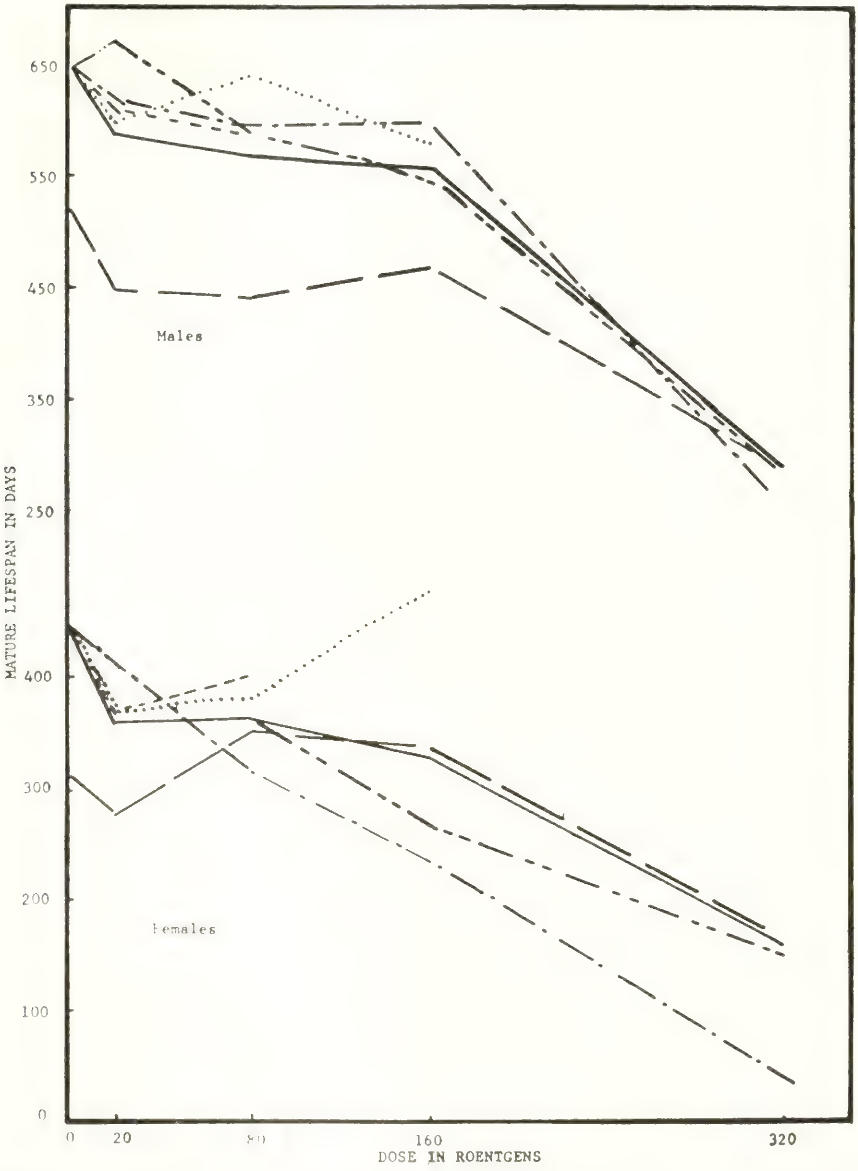

FIGURE 5. Mature lifespans of mice treated *in utero* with 0, 20, 80, 160 or 320 roentgens and plotted for the given embryological ages when the irradiations were absorbed. Embryological ages in days at treatment were designated by the following lines: 6½ ....; 10½ - - -; 14½ — - —; 17½— - - - —; newborn mice — - -, and untreated ——.

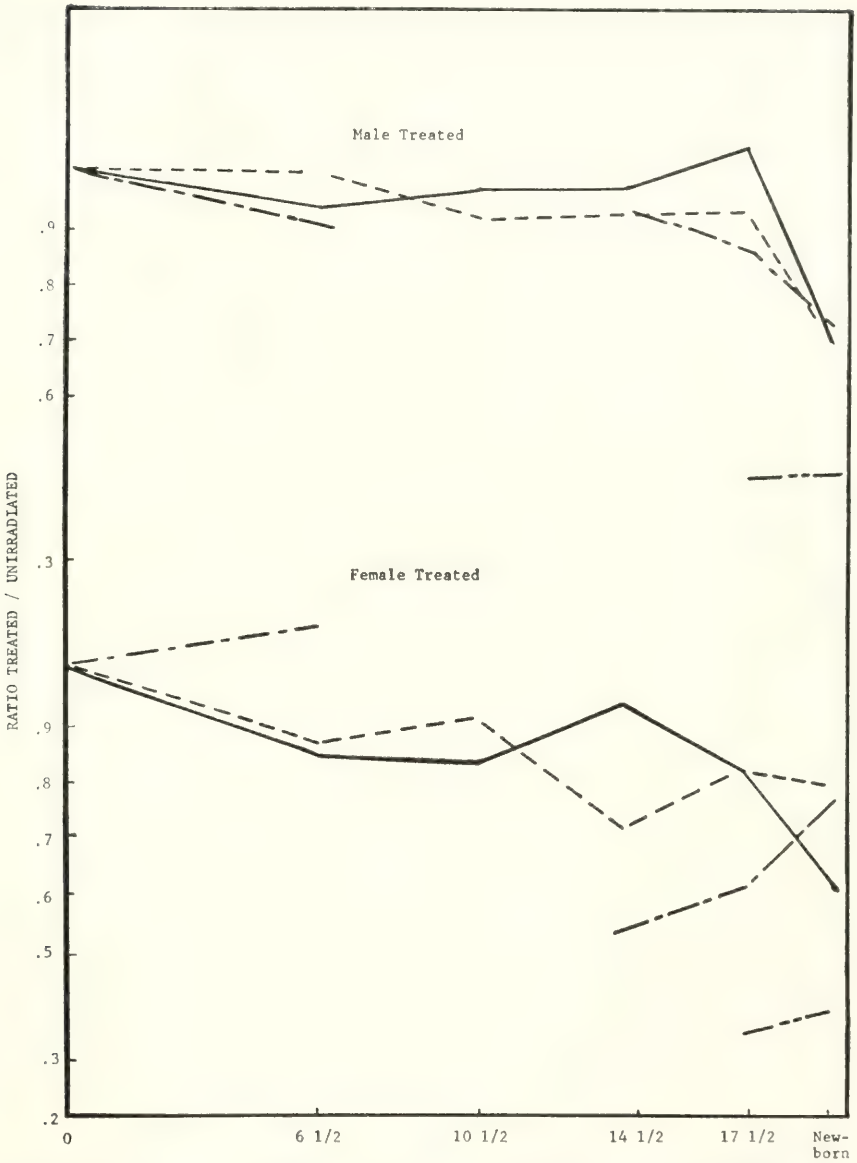

Figure 6. Lapsed days of life between mating to death of mice exposed at different embryological ages to irradiations of given dosages divided by the survivals of the corresponding unexposed mice. The dose of irradiation is indicated as follows: 20 r —; 80 r ----; 160 r —-—; 320 r —--—.

The lower lifespans of the females are evident in all dosages and embryological ages and strains. For the *in utero* treated mice the average unirradiated females survived for only 69% of the mean male lifespan. This male-to-female difference is attributed to the hazards which differentially affect the physiological processes which surround reproductive functions. If the average female life under the different types of irradiation is adjusted for this difference to what would be expected for the male, the female still retains more susceptibility to irradiation than the male. Considering the available data for the dosages taken separately, the 20 r females had 85% of the mature mean lifespan observed for the untreated females, the males 96.7% that of the untreated males. Corresponding comparisons for the other dosages yield for 80 r, 82 vs. 93; 160 r, 73 vs. 89; and 320 r, 30 vs. 43% for females vs. males, respectively.

Comparisons of the sensitivity of the factors which make for longevity in embryos at different growth stages when irradiated are made difficult because all mice exposed to 320 r at 6½, 10½, as well as those in the 160 r, 10½-day embryo age groups, failed to reach the mating age. Only two males and one female lived beyond the 75-day date for mating in the 320 r 14½-day treatment group. These mice survived 31, 478 and 35 days following mating. While these data confirm the sensitivity of these mice to irradiation at these dosages and stages, they failed to give a group by which the effects of irradiation at these dosages and embryological stages may be determined in the reproductive and senescence periods of life. The two males and one female in the 320 r, 14½ embryological age group indicate that the life shortening would be severe.

Figure 6, as the companion graph to Figure 5, brings out the contribution of embryological age to sensitivity toward high energy irradiation. The results are determined as the ratios of mature lifespans of irradiated males or females to those of the unexposed mice of like sex. They are plotted on the embryological ages when the irradiations were received. Full data on the separate sexes were obtained on the 0 r, 20 r, and 80 r for each embryological age. Curves for the 160 and 320 r are broken for the reasons previously indicated.

Unirradiated mice on the average had longer lifespans between mating and death than those which were irradiated. For mice irradiated with 20 r and 80 r the mature lifespans change but little over the span during which embryological x-ray exposures occurred. The female adult lifespans were depressed somewhat more than those of the males. Dosages of 160 r and above tended to further reduce the days the mice survived. When the irradiations reach 160 r at 10½ days embryological development or 320 r at 6½, 10½ days, the development of all mice was interrupted before they reached the mating age. That these early uterine irradiations would have caused severe damage to after-mating life was shown by the fact that the two males and one female which did survive this dose at 14½ days died early, 31 and 478 days for the males and 35 days for the female.

REPRODUCTIVE AND SENESCENT LIFESPANS OF MALE AND FEMALE MICE FOLLOWING IRRADIATIONS OF DIFFERENT DOSAGES RECEIVED AT DIFFERENT PERIODS OF EMBRYOLOGICAL DEVELOPMENT

The mean reproductive lifespans of the males were noticeably longer than those of the females in these data. The comparative differences are brought out in Figure 7.

Figure 7 shows a rather constant relation between the lifespans of female to male mice for those treated with x-rays of 20 and 80 r over the different embryological periods. The severity of the effects at 160 and 320 r for the 6½, 10½ and 14½

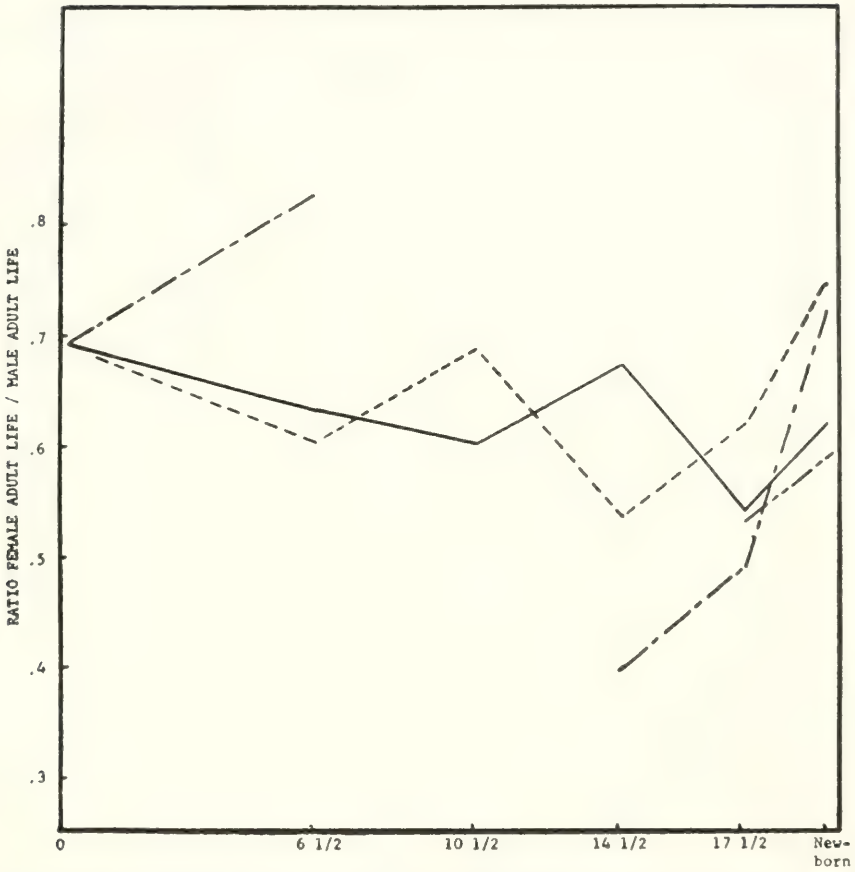

FIGURE 7. Ratios of the mature lifespans of females to those of males for the same irradiation exposures, plotted against the embryological ages when treatment took place. Radiation dosages: 20 r —; 80 r - - - -; 160 r — - —; and 320 r — - - —.

periods of gestation indicate that for higher dosages of x-ray, differences in the lethal or semilethal capacities do appear as embryological development unfolds. The individuals which survive to adulthood bear these unseen effects. The effects were more severe on the female than on the male mice.

ANALYSES OF THE VARIATIONS OBSERVED IN MATURE LIFESPANS

In these data the mature lifespans are regarded as dependent on measurable events occurring some 75 or more days earlier, in the interim conception-to-birth, than when the mature lives took their origin. These events are the characteristics of the basic mouse genotypes and sexes, which compose the mouse population, acted upon by the different dosages of irradiation occuring at specific embryological stages of development in the life cycle. In symbols the equation describing the relationship is of the familiar linear type. The plan of the study as indicated earlier

TABLE III

Duration of mature life as related to exposure to high energy irradiations at different stages of embryonic development

Source of variation	Embryological age (U) in days at exposure to irradiation														
	6½			10½			14½			17½			Newborn		
	df	M.S.	*P*	df	M.S.	*P*	df	M.S.	*P*	df	M.S.	*P*	df	M.S.	*P*
Dose (T)	3	230		2	325		3	1261	(3)	4	6134	(4)	4	2066	(4)
Strain (G)	8	227		8	435		8	1375	(4)	8	895	(2)	8	1640	(4)
T × G	24	624		16	995	(1)	24	564		32	846	(4)	32	808	(4)
Sex treated (F)	1	13718	(4)	1	12171	(4)	1	24748	(4)	1	24098	(4)	1	9344	(4)
T × F	3	398		2	96		3	506		4	430		4	194	
G × F	8	551		8	797		8	292		8	986	(2)	8	226	
GTF	24	547		16	514		24	444		32	403		32	274	
Unaccounted for (E)	72	469		54	548		72	346		90	383		90	216	
Dosage classes	0, 20, 80, 160 r			0, 20, 80 r			0, 20, 80, 160 r			0, 20, 80, 160, 320 r			0, 20, 80, 160, 320 r		

df = degrees of freedom. M.S. mean square ÷ 100. *P* probability, level of significance utilizing the unaccounted for variance (*E*) as the error term for fixed variates. *P* probability levels, 1 = 0.05, 2 = 0.025, 3 = 0.01, 4 = 0.005 or less.

was orthogonal. The biological nature of the results made the results lack orthogonality at several points. However, there are several desirable orthogonal arrangements of the data which give useful information on the effects of the different independent variables on lifespan. We shall present the two analyses which have proved most informative.

The variance constants of Tables III and IV allow evaluations of the aftereffects on mature life processes of mice irradiated some 75 days or one-eighth of their expected lifespan earlier. The mice in these studies have withstood the

TABLE IV

Duration of mature life as influenced by the embryological age when the mice were irradiated with different dosages of high energy x-rays

Source of variation	Dose of irradiation in roentgens											
	20 r			80 r			160 r			320 r		
	df	M.S.	*P*	df	M.S.	*P*	df	M.S.	*P*	df	M.S.	*P*
Embryological age (U)	4	1487	(4)	4	724		3	1255	(2)	1	25	
Strain (G)	8	942	(3)	8	539		8	1110	(4)	8	883	(4)
U × G	32	698	(4)	32	791		24	1044	(4)	8	497	(1)
Sex treated (F)	1	24123	(4)	1	19245	(4)	1	17308	(4)	1	2892	(4)
UF	4	235		4	724		3	1400	(2)	1	7	
GF	8	661		8	662		8	321		8	457	
GUF	32	524		32	698		24	302		8	76	
Unaccounted for (E)	90	328		90	455		72	360		36	210	
Embryological age classes	6½, 10½, 14½, 17½, Newborn			6½, 10½, 14½, 17½, Newborn			6½, 14½, 17½, Newborn			17½ Newborn		

Symbols as in Table II.

physiological consequences over the earlier parts of their lives following the acute embryonic irradiation of different dosages. They are to that extent a group, selected by nature as being resistant. The irradiation effects observed are long-time effects.

The first arrangement of the data (Table III) is analyzed within constant embryological ages, when the irradiations occurred, and the numbers of mice for the different dosages. These data form an orthogonal design. The x-ray dosages given the treated groups are shown at the bottom of Table III. In the 6½ embryological age treatments there were 4 dosages of x-rays, 0, 20, 80, and 160 r, which were complete in having all cells filled in the factorial design. In the 10½-day embryological age group there were 3 dosages which had equal numbers of mice throughout, 0, 20 and 80 r, etc.

Table III shows that differences in irradiation doses, in the strains of mice exposed to the irradiations and in sexes of the individuals treated, all influenced the duration of mature life when the irradiations came in the latter half of pregnancy or in the newborn. Throughout, the greatest influence on the lifespan by a wide margin was the sex of the mouse. However, none of the sex interactions with dose or strains showed significant deviations.

The fact that the variances for dose of irradiation and for strains were comparable to the random variance (E) in the 6½ and 10½ embryological age treatments is not to be taken as indicating these variables have no effect in causing life shortening for the full possible range of the irradiation dose treatments. The 320 r dose so severely affected lifespan as to cause all those exposed to die early. The limitation of studying only after-mating lifespan prevented the irradiations from showing their full effects, as the severely injured mice all die early, *in utero* or shortly after exposure. Similarly, dosages of 160 r at 10½ days of embryonic age also resulted in the early deaths of all the progeny. Actually the irradiation treatments at the 10½-day age were probably the most significant to over-all life. The severity of the effects prevented them from showing in the mature aged group. The interesting fact which comes from these comparisons is that the mice can take the lower dosages at these embryological ages and yet not be affected significantly in their later lifespans, even though higher dosages are so toxic.

The data on the mice treated at 10½ days were self-curtailed as mice exposed to higher dosages than 80 r all died before they reached breeding age. Those treated with 80 r showed less than a 10% drop in lifespan over those which were untreated. The mice treated at 6½ days showed little dose effect. For 80 r there was a 6% and for 160 r only a 4% drop in lifespan. These facts suggest that the dosage effects *in utero* on life's duration after reaching adulthood would be small in the 20 and 80 r range. This point was tested by analyzing the data on the 14½, 17½ and newborn treated mice restricted to those exposed to 0, 20 and 80 r x-ray exposures. These analyses show that these dosages had only statistically insignificant effects although they each lowered the lifespans somewhat, 17%, 14% and 3%, respectively, for the 80 r treatments of the 14½, 17½ and newborn mice.

The data may be arranged to quantitate the effects of treatments at the different embryological ages when the dosages of irradiation are fixed at 20, 80, 160 and 320 r. These data are presented in Table IV.

The mean squares of Table IV show intrauterine treatments reduced significantly the durations of mature life in two of the four dosage treatments, 20 r and 160 r. Examination of the differences from which these variances were calculated shows that the observed significance values depend on the fact that the newborn mice, both untreated and x-ray irradiated, had lower average lifespans than the mice treated *in utero*. Why this should be is not clear. The mothers of these newborn mice were bred and randomly chosen from the same strains and in the same way as those bearing the mice exposed *in utero*. However, omitting this group from the calculations the results show that there were no real differences among mice treated at the different developmental stages, 6½, 10½, 14½ and 17½ days with 20 roentgens. If anything, their mature lifespans were increased.

Mice exposed to 160 r, if males, had their lives reduced in length 8% to 15%. If females, a gain in lifespan of 4% was noted if the mice were treated at 6½ days. A loss of 18% of the control lifespan occurred in the mice treated at 14½ days of embryological age with 160 r. The effects of the x-ray exposure to 160 r at embryological ages 6½, 14½ and 17½ days were significant even when the newborn mice were not considered in the analysis.

CONTRIBUTIONS OF THE DIFFERENT COMPONENTS TO VARIANCE

In the following interpretation it is assumed that the variations in lifespan are due to components acting additively. Each component is regarded as fixed, as contrasted with the assumption of randomness, as it is doubtful if any factor which is subdivided into less than 10 to 15 parts, even though selected at random, can really be considered as random in the sense of representing the whole biological population of a species or even the laboratory mouse. The considerations lead to the general equation portraying contributions of the variables influencing lifespan, Y, as

$$Y_{ijkl} = u + g_i + t_j + (gt)_{ij} + f_k + (fg)_{ik} + (ft)_{jk} + (fgt)_{ijk} + e_{ijkl}$$

where u = the overall mean; $i = 1, 2, \ldots, 9$, the inheritance types; $j = 1, 2, 3$, (or 4 or 5), quantities of irradiation; and $k = 1, 2$, the sexes exposed to irradiation. The strain differences are considered as partial measures of the genotype effects which are omnipresent in most biological populations. In general they represent gene effects. However, they by no means represent the whole effects of the inheritance. Even in these data, sex differences are genotypic effects but are attributable to gene influences or their equivalent, transmitted generally as whole chromosomes, and developing their effects through internal balances throughout the whole organism. The component analyses of the different variances in Table III, with given embryological ages constant, are as shown below.

Source of variation	d.f.	Components of variation
Among genotypes	8	$E + 2_jG$
Among dosages	$(j - 1)$*	$E + 18T$
Genotype × dosage	$8(j - 1)$	$E + 2GT$
Between sexes	1	$E + 9_jF$
Sex × genotype	8	$E + jFG$
Sex × dosage	$(j - 1)$	$E + 9FT$
Sex × genotype × dosage	$8(j - 1)$	$E + FGT$
Unaccounted for variation		E

* j = 3, 4, or 5, depending on which embryological age is analyzed.

The components can be interpreted as follows: *G* is the variation due to genotypic or hereditary differences as partially measured by strain differences. *T* is the variation due to differences in effects of the dosage levels, and *F* is the genotypic variation induced by the sex differences in the lifespans under the conditions of irradiation. The interaction terms are interpreted as arising from the differential responses of the genotypes of sexes from one level of irradiation to the next. The term, *E*, is considered due to uncontrollable variation, and represents random variation of individual differences of mice of the same sex within a litter given the same treatment.

The chart at the top of Figure 8 gives the graphs showing the contributions of the different components of variance plotted on the embryological ages when the irradiations took place. Interactions of *FG*, *FT* and *FGT* are combined, as they are small, and designated *I*. A similar analysis of the variance contributions, where the constant element is the irradiation dose and the embryological age becomes a variable, is shown in the graphs at the bottom of the figure. Again the interactions *FG*, *FU* and *FGU* are combined and represented as *I*. Between these two charts an appreciation may be gained of dosage (*T*) and embryological effects (*U*) as well as those of genotype (*G*), and sex (*F*) and their interactions (*I*). The scale of the ordinates is percentage of total variation which is contributed by these various components.

The differences in the capacities of the two sexes (*F*) to receive irradiation at different stages in embryological development and later perform the functions normal to life are shown to have the most effect in altering mature lifespan for the mice under investigation. These effects hold whether the irradiations are at early, medium or later periods in embryological development. They hold for all dosages of radiant energy here under investigation as shown in the lower chart.

The next large contributor to variance under the conditions as shown in either chart is the variance made up of the effects of many factors, most of which, even in the best controlled environments, are still environmental in origin, *E*, the uncontrolled variance. This variance is most important in the early embryological stages and lower dosages of irradiation.

Genotypic influences on mature lifespans (*G*) are materially influenced by the time in embryological age and the level of irradiation dosage when they are considered. In the 6½ and 10½ embryological age groups the genotypic differences, as marked by the strains, have little effect on the later lifespans. Similarly, the strain differences show little effect if the dosages of irradiation are only 20 or 80 roentgens. The low values of these genotypic effects may be due to two causes. The anatomical and physiological structures of the embryos through 10½ days may not have differentiated the genetic elements, or the time sufficient for them to reach a size for radiant energy to produce apparent effects on life. Similarly, irradiation dosages of 20 and 80 r received in the embryological stages may be insufficient for them to affect processes significant to mature lifespan.

By 14½ days gestation the genotypic effects have begun to express themselves. They continue to show their effects in the embryos at 17½ days and in newborn mice. The effects are on the order of 10%. They become increasingly important to animals irradiated with larger dosages, increasing from 7% for the 160 r to 26% for 320 r treated mice.

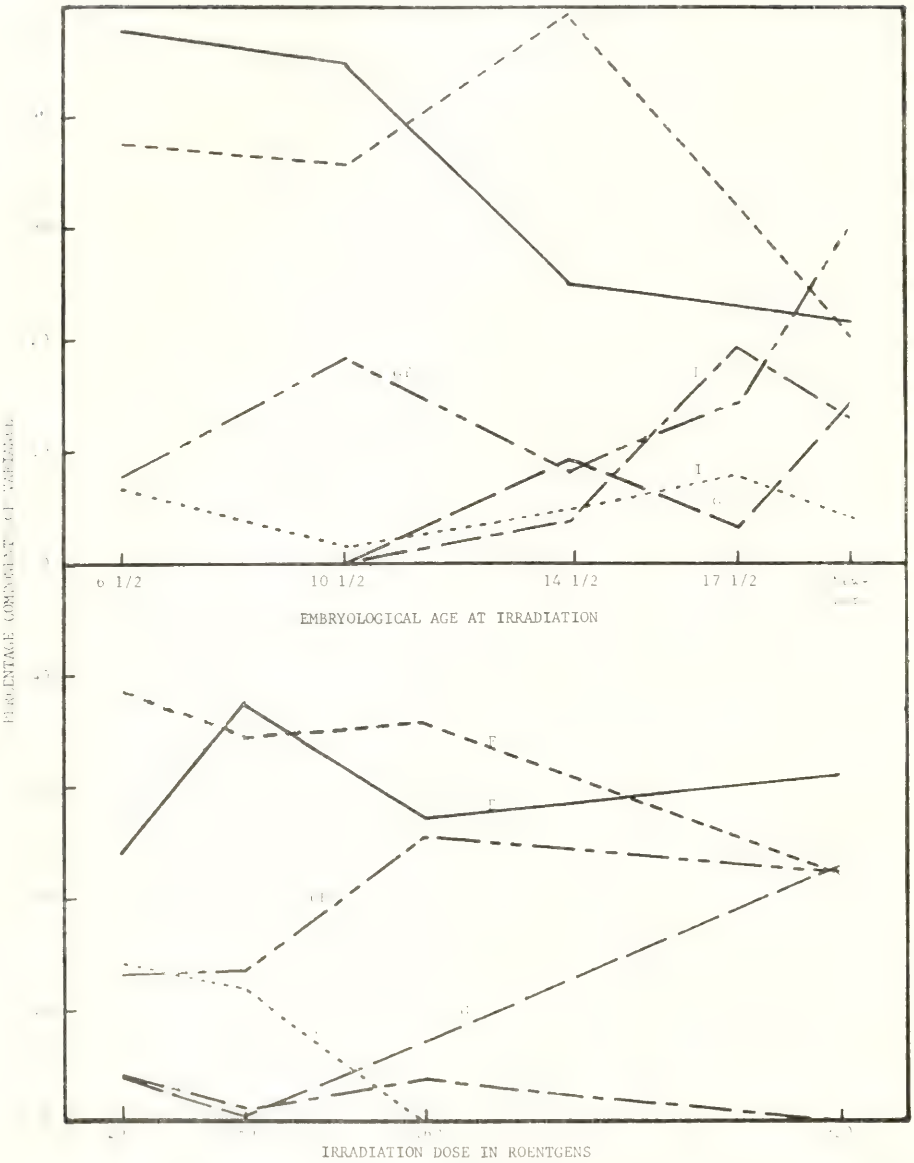

FIGURE 8. Contributions to variance of mature lifespan in these studies, upper chart, by genotypic and sex differences in strains of mice following *in utero* exposures to specific x-ray dosages received at different stages in embryological development. The effects of the different components are shown by the following lines; Genotype (G) — — —; x-ray dose (T) — - —; interaction (GT) — - - —; Sex (F) - - - -; combined sex interaction (I) . . . .; and unaccounted for variations generally of environmental origin (E) —. Lower chart, the variance evaluations are distributed on x-ray dose received; G, F, I and E have like significance to those shown by the same lines in the upper chart. The effect of stage in embryological development when irradiation was received (U) is shown by — - —; and the (GU) interaction effects by — - - —.

Genotype also interacts with both irradiation dosage (*GT*) and with stage in embryological development (*GU*). These contributions to variance range from 10% to 30%.

Individually the embryological stages when the irradiations took place have relatively small percentage effects on the mature lifespans, 0–5%, although they do have pronounced effects on the juvenile survival.

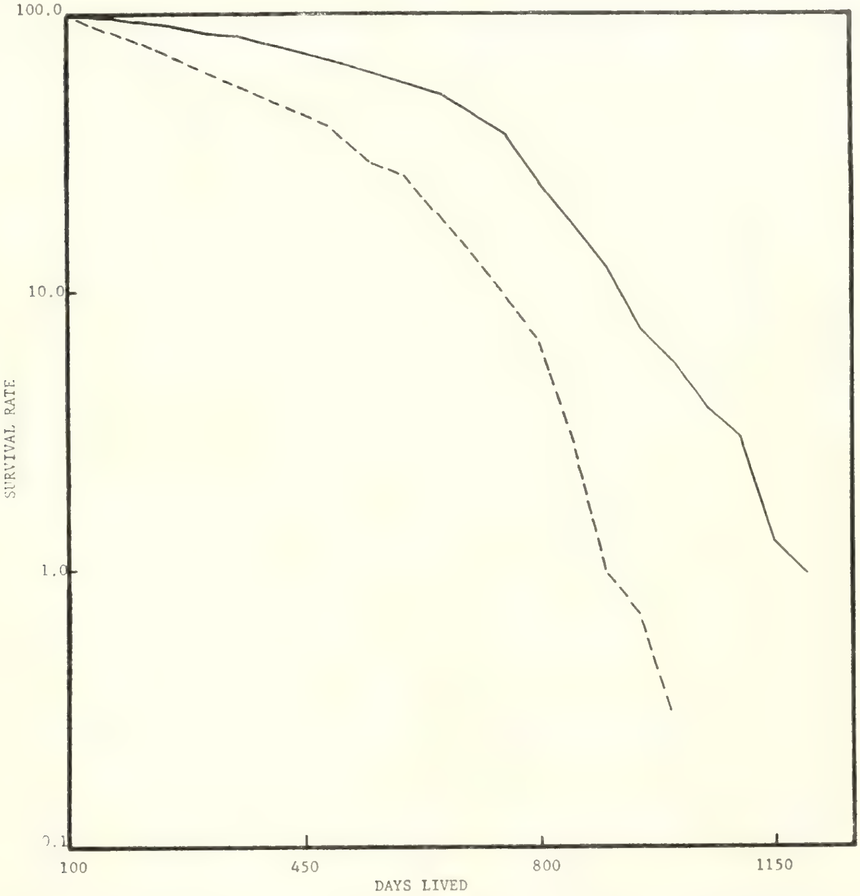

Figure 9. Survival rates of male and female mature mice exposed to various dosages of irradiation during uterine development. Males —, Females - - -.

There are three different interactions which include sex of the treated mouse in each of the different embryological or dosage treatment groups. Individually these effects are small and by statistical standards insignificant. These interactions are combined in the lines marked *I* on the charts. They approach uniform values throughout the different treatment groups. Their total contribution to variance range is between 0 and 15%.

SURVIVAL CURVES OF MATURE MICE FOLLOWING EMBRYONIC IRRADIATION

The survival patterns of these mice may be brought out more fully in life curves showing the proportions of mice surviving at successive ages over their known life-spans. Figure 9 gives these data separately for the males and females in the populations. The numbers of males and females in each group are equal.

Male survival rates are at all ages greater than those of the females. At the stage when the population has reached only 1% of those which started as mature mice at 100 days, the males have lived 300 days more than the females. As pointed out in the earlier analyses the greater part of the survival rate differences of the sexes is attributable to physiological differences in the reproductive functions rather than to the irradiations. The form of the male survivals is like that of the females in showing noticeable curvature with age, indicating that no one agent is responsible for the life changes.

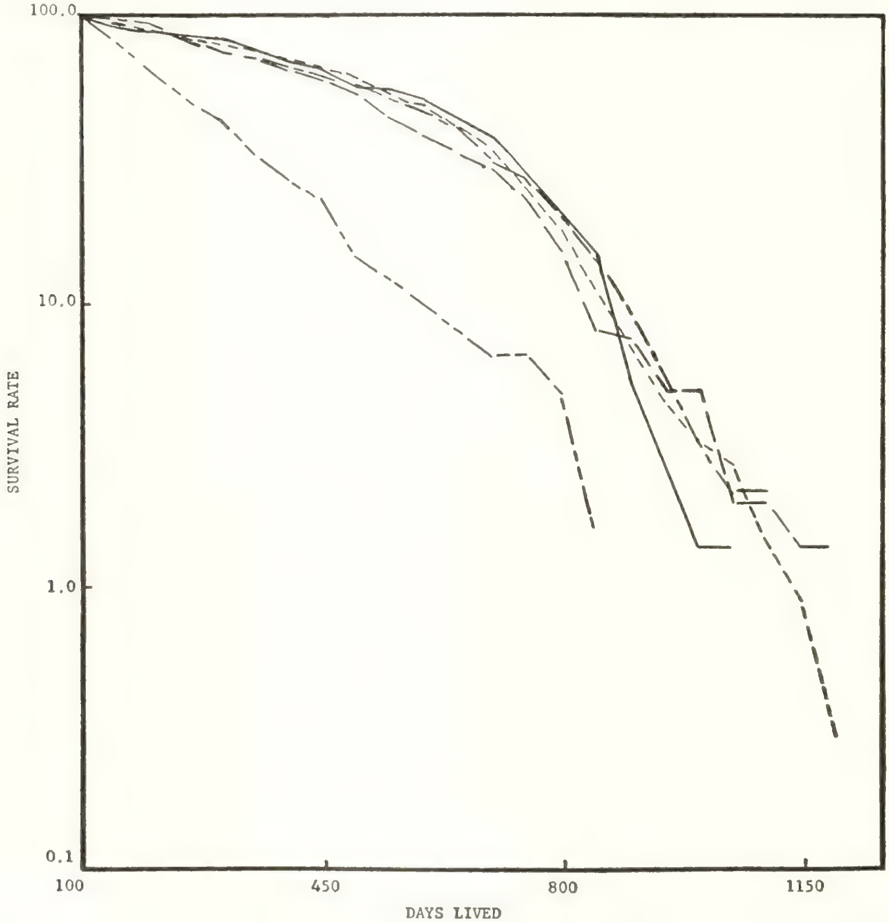

FIGURE 10. Survival rates of mature mice exposed *in utero* to different dosages of irradiation. Zero roentgens —, 20 r - - -, 80 r — - —, 160 r — —, and 320 r — - - —.

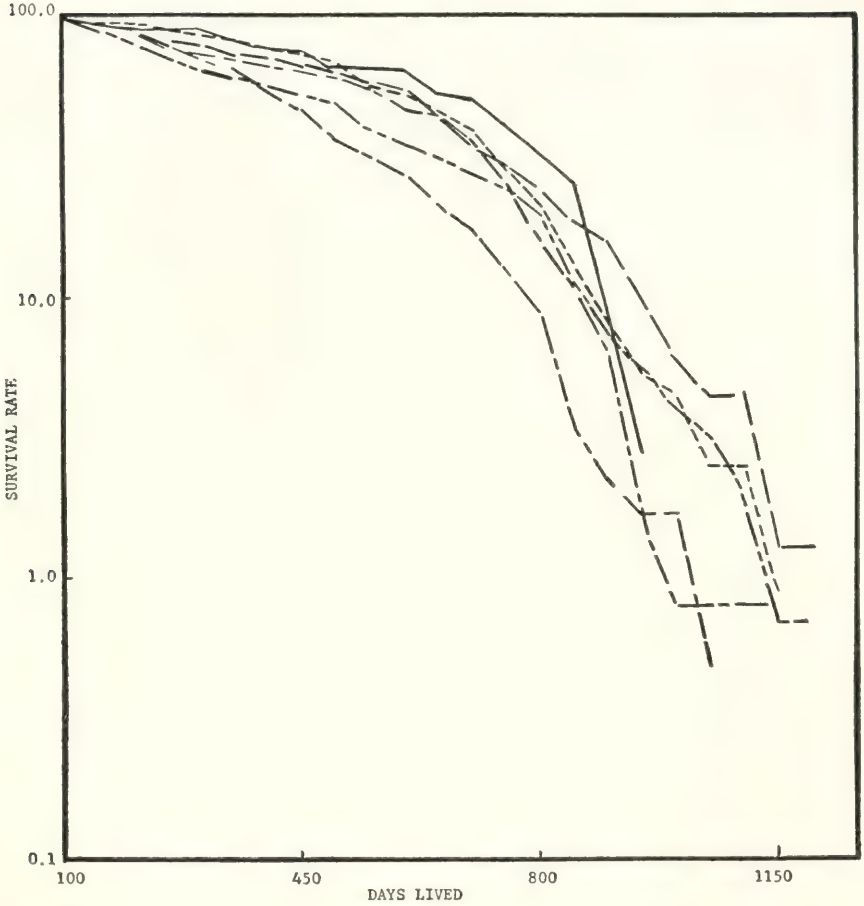

FIGURE 11. Survival rates of mice exposed to x-irradiation at different stages of uterine development Untreated —, 6½ days gestation - - -, 10½ days — —, 14½ days — - —, 17½ days — - - —, and newborn — — - — —.

Figure 10 arranges the data to show the survival rates for the different x-ray dosages. For these data the sexes are balanced. Survival rates are shown for the 5 dosage treatments.

The survival rates for the 0, 20, 80 and 160 r are all smoothly flowing curves convex to the age axis for the mature mice. Deaths are few at the beginning of maturity. They increase and become more frequent as age advances. When 50 of the cohort of 100 have died, the survival rate differences are not large but they are in order of the x-ray dosages the mice received *in utero*. At 850 days of age the unirradiated control mice show a decrease in survival rate of unknown cause. In general, for those mice reaching adult age, the survival rates are fairly comparable in the range of x-ray dosages of 0 through 160 r.

Changes induced by 320 r irradiation while the mice are still *in utero* have permanent effects on survival even when the mice reach young adulthood. The rate of survival takes the form of a simple exponential instead of the convex form of the lesser dosages. Each day of life is accompanied by a constant percentage decrease in survival. The chart makes it obvious that above 160 r the effects of x-radiation on adult lifespan increase rapidly. For 160 r, 10% of mice remain alive at 835 days while with 320 r, 10% survival is attained at 600 days or 72% of the lifespan reached by the lower irradiated mice. The detrimental actions of 320 r x-ray acute irradiations resulted in pronounced lowering of adult lifespans. These losses in expected lifespan occurred even though the mice previously had passed through an earlier intense selection period eliminating those so susceptible to irradiation as to die *in utero* as in the juvenile period of development.

Figure 11 gives the survival curves of these mice distributed by the stage in embryological development when the x-raying took place. The curves cover a fair range although not as great as that observed for the extreme x-ray dosages. At the point where 50% of the mice in the original populations had died, the untreated mice had the longest survival; the 6½- and 10½-day treatments were next and about equal to each other; the 14½-day treatments showed slightly less survival, 17½-day noticeably less and the newborn least of all. The sequence of the survivals again comes from the fact that the severely detrimental actions of 320 r and 160 r at some embryological ages have destroyed the original orthogonal character of the experiment. For the 10½-day period only, the mice treated with 0, 20 and 80 r reached the beginning of the adult stage. Similar selective action occurs for some of the 6½- and 14½-day treated mice. Some irradiated mice can live for long periods.

In each irradiated group 1% to 7% of the group lived longer than the unirradiated controls of this study. All of the curves for the mature age lifespans for mice irradiated at the different embryological ages are convex to the age axis, indicating that the mouse characteristics operated on by the radiant energy are numerous and interacting.

DISCUSSION

Lifespan is a variable made up of many composite parts, each of which must integrate perfectly with the other, as well as the environmental factors, to bring it to its full expression. It is the variable which is most likely to measure quantitatively on a single scale the embryological, physiological and morphological damage accumulated by the exposed animal.

Somatic cells arising from the germ line have equal opportunities for genetic change. Without any wastage it takes some 43 cell generations (successive divisions) to develop a mouse. This number is beyond our grasp, 2^{43} for each individual. During these divisions there are many opportunities for gene mutations and chromosome aberrations to occur in the same way as they appear in the germ line.

Mutations notoriously require conditions of proper embryological development for their effects to receive phenotypic expression. Different thresholds of change appear throughout life but the right one must be reached for the appearance of characteristic effect of the given gene.

Aberrant phenotypes appearing in populations of mice receiving irradiation during embryo development likewise follow this step-like pattern. The aberrant phenotypes observed for the same radiation dosages given at different *in utero* stages show changes characteristic for expected stage of development when irradiation took place. Seldom do they show changes related to those naturally appearing in later development. The threshold pattern points to necessary specific conditions in both host and treatment for the appearance of the effect. The ultimate causes of the changes in physiomorphic development affecting lifespan seem best sought from this viewpoint, rather than in hypotheses generated through indistinguishable lumping of causative components into single constants to form one of the many curves which may be theoretically generated, *e.g.*, the Gompertz curve.

The direct effects of the irradiations on the exposed cell may be on the genes, the chromosomes, the action links between the genes and chromosomes, the cytoplasm, or cytoplasmic elements. With the large numbers of ion pairs released per roentgen it seems impossible to think that any cell often escapes receiving at least one and often many when irradiated with the large dosages usually employed. Yet the evidence shows that sometimes cells do perform their functions even when they have been exposed to high dosages of radiation. It appears that cells have escape mechanisms for reducing the customary irradiation damage which may be immediately effective, or effective after an interval of further development. There are several categories into which these radiation effects may be classified. The growth pattern of the cell may be so altered as to make its future cells function abnormally with regard to the organism as a whole. Other cells may stop cell division, restricting an organ system essential to the whole. Subsequent growth processes may be so ineffective that a new event must occur if the damage is to be repaired. In the latter category may be included some gene or chromosome aberration—generated phenotypes. Chromosome aberrations initiated by breakage, on the other hand, may rejoin and the cell recover its previous normal functions. Differential cell growth where the irradiation damage is "healed" through replacement of defective cells by others of normal constitution, as in wound healing, offers another means of arriving at a normal phenotype. However useful this mechanism is, it is not recovery of the irradiation-damaged cell but should be labelled one of cell replacement. Cytoplasmic elements affected by radiation frequently are not carried through future mitoses or are incapable of reorganization. They offer recovery mechanisms for their repair.

Data on all types of cells seem to indicate that the greater parts of their volumes are not occupied by radiation-sensitive structures. Rather they allow the radiation to pass through, or, when absorption occurs, are unaffected.

Earlier studies on malformations following ionizing radiations were concerned primarily with effects that were observed in embryos some time between the time of irradiation and shortly after parturition (Bagg, 1922; Job, Leibold and Fitzmaurice, 1953; L. B. Russell, 1950; W. L. Russell, 1954; O'Brien, 1956; and Rugh, 1953, 1959). The results generally agree that the type of malformation depends both on the level of irradiation and the embryological stage of development during which irradiation took place. Most exposures were to high dose acute irradiations causing cell damage far beyond the physiological limits the exposed animals might repair.

Additional studies in recent years have attempted to evaluate the long-term sequelae of embryonic or fetal irradiation. It has become evident from these investigations that although an animal exposed to embryonic irradiation may appear normal at birth, effects on morphological and behavioral characteristics may have been produced that become noticeable in later life, as cataracts (Stadler, 1959; Gowen, 1963) or volitional activity (Huff and Gowen, 1960). In some instances changes following embryonic irradiation have been found both by histological study and by functional tests of the organ system under study. Beaumont (1960, 1962), in quantitative histological studies of the ovary and testis in rats, demonstrated a deficiency in germ cells following embryonic irradiation. Several workers, including Rugh and Jackson (1958), Russell *et al.* (1959), and Nash and Gowen (1961), observed in mice long-term effects on the reproductive system in that adult reproductive capacity had been altered following embryonic irradiation.

Effects of prenatal irradiation upon postnatal growth have been reported for several species, including the mouse (Russell *et al.*, 1959; Nash and Gowen, 1962), the rat (Ershoff and Bavetta, 1958), and cattle (Parish *et al.*, 1962). In several instances effects of prenatal irradiation upon growth did not become evident until some time after treatment. Nash and Gowen (1962) noted, for example, that certain embryological treatments did not produce a noticeable effect upon body weight until some two months after treatment. Similar "delayed effects" also have been reported for certain behavioral effects. Rugh (1956) found that mouse embryos irradiated with doses of 5 to 300 r exhibited more nervous excitability than controls when tested at 15 days and two months of age. In some cases behavioral changes have been observed in later life although no distinct morphological malformation or lesion could be demonstrated. It is evident from these examples that there may exist changes induced by embryonic irradiation which may not have a noticeable effect until late in life.

Although ionizing radiations have been widely used as an experimental means to accelerate changes sometimes associated with aging in mammals (Gowen, 1962), the exact relationship between lifespan and other physical and biological variables is still not clear. Attention has been directed towards changes progressive with age, sex, genetic background, and radiation dose response. Abrams (1951) found that the effect of irradiation on survival decreased rapidly with increasing embryological age and increasing age after birth. Similar results were reported for the rat by Stearner and Christian (1951), who observed that radioresistance increased rapidly between one and 48 hours after birth so that by two days of age rats were only slightly less radioresistant than adults. Although some workers (Sacher, 1947, 1957; Blair, 1956) have postulated that radioresistance should probably decrease in some uniform manner with advancing age, other workers, including those in our laboratory, have found periods of relative stability of radioresistance. Kohn and Kallman (1956), for example, found that the $LD_{50/30}$ was a linear function of age from 37 to 105 days of age, but remained nearly constant from 115 to 709 days. Spalding and Trujillo (1962) noted in mice irradiated between two and 21 months that radioresistance was relatively stable through the first half of adult life but then declined quite rapidly. Sacher (1957) also found little dependence on age and mean accumulated dose to death of animals irradiated between 100 and 600 days.

There have been few systematic studies concerned with the effects of *in utero* irradiation upon adult lifespan. Zuikova *et al.* (1959) reported that in dogs irradiated during the last trimester of pregnancy, the duration of lifespan was in direct relation to dose. Russell *et al.* (1959) found that postnatal viability to 36 days of age in mice was lowered in animals irradiated with 200 r on days 9½, 7½, or 11½. In long-term survival, of the data available at that time, lifespan appeared reduced in those mice that had been exposed to 200 r on days 7½, 11½, or 13½.

Considering the four embryological stages used in this investigation, 6½, 10½, 14½, and 17½ days, and the levels of irradiation, through 160 r, it is difficult to discern clear-cut "critical periods" for the induction of changes in adult lifespan such as have been described for several other characteristics. Instead, noticeable effects on lifespan appear to be a result of interaction of a certain radiation dose with a certain specified embryological stage. The embryological stage at which embryos were irradiated did not seem to influence adult lifespan as long as the dosage did not exceed 80 roentgens.

At the higher dose level the exceptions occur in female mice treated at 14½ and 17½ days gestation. After 160 r exposures life shortening occurs at a more rapid rate than expected from the previous trend in lifespans observed in 0, 20, 80, 160 r treatments. These changes are indicative of a threshold effect coming in this region of dosage, initiated by the radiation absorptions occurring in a more resistant set of receptors as well as those already taking up the radiant energy.

The consequences of acute irradiations have generally been studied as changes closely following irradiation as expressed in different morphological types, morbidity and rapidity of death in 30 days. The data herein show that as far as duration of adult lifespan is concerned, the effects may be carried quiescent through to expression over a fairly wide age span in later life. In this sense the late effects become comparable with more limited characters such as cataracts following irradiation, but on a different time scale. The shortening of life is the consequence of the experience through which the mouse passed at some embryological stages of development. The cataract stimulation, although having a long quiescent period of 300+ days during which no changes were evident, was dose-dependent and results from acute irradiation dosages occurring at 46 days from the birth of the animal when most of the embryological pattern of development was complete (Gowen, 1962).

The nature and mode of action of the changes induced by embryonic irradiation that result in a shortening of the lifespan are still speculative. There is increasing evidence that aging may be caused by the gradual accumulation of spontaneous chromosomal rearrangements and mutations in the somatic cells of the body (Gowen, 1934, 1962; Curtis, 1963). Through several methods of controlling chromosome aberrations it has been shown that their frequency was inversely proportional to the life expectancy.

Teratogens are also capable of producing chromosomal anomalies in various tissues. Mustard gas acting on sperm of *Drosophila* furnishes one of the early examples (Crowe, 1961). Ingalls *et al.* (1963) demonstrated that a teratogen, 6-amino nicotinamide, when administered to pregnant female mice about the 13th day of gestation, produced chromosomal anomalies. Cells exhibiting polyploidy and fragmented chromosomes were observed not only from tissues adjacent to palatal defects but in tissues remote from the palate as well.

In the present study, embryonic x-irradiation offers a means of producing chromosomal aberrations in the developing embryos. Although some of the embryonic cells may harbor mutated chromosomes they may function in a normal or near-normal fashion. Others may cause marked changes in body maintenance with a subsequent curtailment of life. A wide range of change leading, however, to like as well as different effects, may be induced giving high variability even within progenies of given inbred litters.

A major concern of the present investigation was to evaluate the contribution of genetic background on lifespan in response to *in utero* irradiation. Although mammals, and the mouse in particular, have been widely used in radiation and aging studies, there is a surprising lack of information on lifespans in mice born of different mating systems. Inbred and hybrid mice, such as were used in this investigation, provide insight into the basic radiation responses over the period when aging is occurring. Several studies in the past have demonstrated that genetic constitution is a major factor in adult lifespan. Gowen and Stadler (1956) utilized 10 genetically differentiated strains of mice to study the effects of irradiation at 40 days of age upon lifespan. The study included the three strains reported in the present paper. Genetic differences were found to influence the effects of x-rays throughout the acute dose range up to 960 roentgens. Genotypic strain differences were observed by the expression of different radiation syndromes. Chai (1959) also observed genetic differences in lifespan. In general, hybrid mice had longer mean life and lower mortality at earlier ages than those of the parental inbred strains. This may be due to the hybrids having a greater reserve of cells unaffected by such environmental agents as radiation on which to draw for life maintenance (Haverland and Gowen, 1960).

Differences among inbred strains considered to be primarily genetic in origin, have been noted by Henshaw (1944), Grahn (1954), Gowen and Stadler (1956), Haverland and Gowen (1960), Kohn and Kallman (1957), and Grahn and Hamilton (1957). Progress has been made towards understanding the nature of the genetic and physiological factors conditioning the radiation response. Kohn and Kallman (1956) reported that in the strains of mice employed by them, greater sensitivity was a result of recessive genetic factors. Grahn (1958), in crosses involving the least and most resistant strains from a series of lines, estimated the genetic mean of the LD_{50} to be about 50% of the total variance. Presumed single-gene differences in radiation response have been observed by Doolittle (1961) and Bernstein (1962).

Stadler and Gowen (1957a, 1957b, 1957c) studied the effect of three body regions on radiation response in five inbred strains of mice, as measured by percentage survival and length of survival. Differences between responses of strains were noted at different levels of irradiation. The extent of the interactions among x-ray doses and strains was large, indicating that each strain had a distinctive pattern of radiation response.

In the results of the effects of embryonic irradiation upon lifespan reported in the present paper, a quantitative examination of genetic influences was provided by a variance analysis and a breakdown of the variance into its component parts. The variance analysis revealed that strain differences influenced the length of adult life when embryos were exposed to irradiation during the latter part of gestation, from 14½ days on. This would indicate that by this stage in development genetic differences have been established sufficiently well in the mouse so that irradiation produces differential responses, as measured by adult lifespan. There are indications from other studies (Rugh, 1958, 1963; Nash and Gowen, 1963) that genetical differentiation, as measured by radiation response, may be expressed even earlier in embryological development. The lack of significant strain differences in the dosage sequences following irradiation at 6½ and 10½ days is a reflection of the toxicity of lower acute x-ray dosages received by these strains. When the embryos have passed these thresholds in development the influence of strain differences on subsequent life becomes less.

Of particular interest in the present data is the presence of significant dose-by-strain interactions following irradiation late in pregnancy. This indicates that the different genotypes are responding through different physiological channels over the range of radiation levels. Interactions of this sort following embryonic irradiation were also reported for postnatal growth by Nash and Gowen (1962). In the present study genotype-by-treatment interaction accounted for up to 30% of the total variation in adult lifespan. The existence of this type of interaction furnishes an excellent tool for further understanding of the basic radiation response.

The possible existence of genetically determined variation in the timing of embryological development, as indicated by this analysis, lends biological significance to the mechanisms of radiation-induced lifespan changes. The early work of Painter (1928) and Venge (1950) demonstrated that differences in size between Flemish-giant and small Polish rabbits could be traced back to early embryonic stages and differences in cleavage rates of fertilized ova between large- and small-sized breeds. Mechanisms having these qualities offer means of understanding how they and other genetic differences may appear in radiation effects on later lifespan and other effects.

The evidence from Nash and Gowen (1962) shows that these mice irradiated *in utero* were reduced in size following irradiation. The growth reductions were dependent on both dose and stage in embryological differentiation. The weight changes to 75 days were small for the 20 and 80 r treated mice. They became more severe at 160 r, particularly for the 10½-day irradiations where at birth the mice were but ⅔ size and unable to survive to the 12-day age. The 75-day weights were consistently reduced following 320 r irradiation.

These changes can be related to observations on like actions by other agents which result in the separation of blastomeres, or cutting of Planaria (Morgan, 1898) into small pieces which on regeneration result in separate animals of small size. The x-ray action seems to come from reducing the numbers of certain vital cells. It is as though there was, for each egg type, a limit to the total cells which may divide. In eggs where the blastomeres lose their totipotence, the loss becomes that of specific cells, precursors of specific organs. In this case these organs may be affected, giving the appearance of specificity to the abnormalities observed in mice irradiated at fixed stages of development in the embryological sequence.

Schaible (1959, 1963) has studied the sequence of melanocyte distribution in mice and chickens of different genotypes. These cells distribute to eight centers of the mouse coat. Whiteness can be the result of absence, malfunction or death of the pigment cells. The distribution of the pigment leaves a record of the events through which the cells have passed in developing their final products. In general, changes in pigment cell density within the normal sequence do not influence organizing cells to promote cell death. The products are left to reveal patterns of organogenesis. The observations become more complete than for other cells as in the organization of the nervous system where damage or loss of neuroblasts (Rugh, 1962) may remain obscure for lack of visible signs.

The outward spread of pigment from eight centers is revealed in three ways: pigment spots on unpigmented background; absence of pigmented cells in a pigmented background; and the presence of a spot of different-colored hair in the coat, representing the descendant cells of a primordial melanoblast which had changed to a genotype different from that of the original zygote from which it started. The pattern of melanocyte descent offers possibilities of control of the end products of development by both genetic and environmental factors. The factor interactions furnish a significant model for the variations observed spontaneously and under x-radiation in the lifespan of this study. The coat color model displays differences between inbred strains of different genotypes ranging from zero to 90%. Substitution of different genes gives a staircase type of ascent to the frequencies of change within the mouse populations. Genotypic species changes are marked by even broader differences in the timing of the periods when hair or feather follicles may attract melanocytes: in the mouse all follicles of the coat seem to mature at one time and exhaust the epidermal supply of melanoblasts; in the chicken the epidermal supply of melanoblasts does not exhaust, at least in the growing animal.

In this model, tests of the pigment mosaics show they are, in large majority, somatic rather than germinal, agreeing with the evidence on the lifespans of mice under continuing low rate irradiation (Stadler and Gowen, 1963; Gowen and Stadler, 1964). There were no whole-body reversions of coat color in 5165 offspring nor did wild type segregates on test have unexpected gametes. Changes appear to occur only after at least one primordial pigment cell has established.

X-radiation with 150 r to the region of the gravid uterus from ½ to 11½ days, following copulation, in day intervals, revealed two significant high points in mosaic frequencies of the progeny, 2.5 and 10.5 days *in utero* development. However, the acute dose administered had only slight influence in the total mosaics observed over the full period of the irradiations. The suggestions of periods of embryological development of greater susceptibility to acute x-ray dosages, and the lack of pronounced changes in frequencies of mosaics from the unexposed controls, again model what was observed in our data on survival in after-life for the lower dosages of acute x-ray irradiations.

SUMMARY

1. Mature lifespans, 75 days of age to death, when the mice were exposed to irradiations at different stages of uterine development, showed reductions in mean longevity which were dependent on both x-ray dose and period of embryological cycle. The design for this study was factorial. There were five irradiation

treatments, 0, 20, 80, 160 and 320 r, six embryological stages: untreated, 6½, 10½, 14½, 17½ and newborn (19½) days of uterine development, two sexes and nine mouse strains. Three of the inheritance groups were inbred strains and six were the reciprocal crosses of these strains. Efforts were made to obtain two mice for each cell of the design. However, some treatments were so severe that this was impossible. X-ray dosages of 160 and 320 r at embryological ages 6½, 10½ and 14½ days were too lethal to obtain the requisite number of progeny in these groups.

2. Six hundred and forty-seven completed mouse lives were collected. Mature lifespan ranged downward from 1194 days for the males and 921 days for the females. Both of these long-lived individuals had been irradiated, the male with 160 r at 14½ days embryological development and the female with 80 r at 10½ days embryological development. The frequency distributions of lifespan were of several types for the different groups, depending on the treatments, strains or sexes of the individuals.

3. In terms of changes in mature lifespan, sex differences in the mice resulted in marked differences in the mean days they survived. Considering the *in utero* treated mice, the average unirradiated females survived for only 69% of the mean male lifespan. This male-to-female difference cannot be attributed to irradiation effects but rather to the hazards which differentially affect the physiological processes which separate the lives of the sexes, chiefly those associated with reproduction. Adjusting for these effects shows that the average female life under irradiation still exhibits greater sensitivities to irradiation *in utero*. Considering the available data for the dosages taken separately, the 20 r females had 85% of the mean lifespan observed for the untreated females, the males 96.7% that of the untreated males. Like comparisons for the 80 r were 82 vs. 93; for the 160 r, 73 vs. 89 and for the 320 r, 30 vs. 43.

4. Intrauterine irradiations, when limited to 20 to 80 r, reduced the female durations of life at all stages of embryological development more than they did those of the males.

5. Variance analyses emphasized the importance of sex as a controlling element in adult survival when the mice are under pressure of reproduction.

6. Genotypic differences, as measured by strain and as separate from sex, have little effect on subsequent mature lifespans when the irradiation is small in amount, 20 including 80 r, or when given at 6½ or 10½ days of embryological development. The amount of radiant energy absorbed becomes more important to mature life's duration when the dosages of radiation are larger, 160 including 320 r, or when the exposures occur in the latter half of pregnancy. The variance analyses support the significance of the differences on which the above conclusions were based.

7. Genotype also interacts with both stage of embryological development when irradiation occurred and with the dosage of x-rays absorbed.

8. Individually the embryological stages when the irradiations took place have but small percentage effects on mature lifespan, although they do have pronounced effects on survival in the juvenile stage. All changes in lifespan come as direct irradiation effect to the soma of the embryos, as caused by acute irradiation. Lifespan lowering comes largely in the 320 r treated embryos in the

form of a threshold effect which then carries through to the adult as cryptic damage to appear later in life.

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VARIATION OF HEMOCYANIN CONCENTRATION IN THE BLOOD OF FOUR SPECIES OF HALIOTIS[1]

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Hemocyanin is a copper-containing protein found in solution in the blood of certain arthropods and molluscs. It combines reversibly with oxygen, becoming blue when oxygenated, and is commonly regarded as a respiratory pigment. Hemocyanin has been recognized for about 100 years, and numerous investigations of its chemistry have been carried out (Manwell, 1960; Prosser and Brown, 1961). In a consideration of the function of a body constituent it is important to have available data on the quantity or concentration of the substance in the body. Data on the amount of hemocyanin actually present in the blood are, however, scanty. Redfield (1934) collected, from several sources, data on the oxygen capacity of the blood of several molluscs, and from these calculated values for the copper content of the blood. From the copper content and the known composition of the hemocyanin from these species he calculated the hemocyanin concentration in the blood of four molluscs. The values obtained, expressed as per cent, were: *Octopus vulgaris,* 5.9–9.1; *Loligo pealei,* 7.2–8.8; *Helix pomatia,* 1.5–3.9; and *Busycon canaliculatum,* 3.7–6.6. The greatest range of variation found in any one species was about 2.6-fold. It may be calculated from data on the copper content of the blood of two of these species (Prosser and Brown, 1961) that the hemocyanin content of *Helix pomatia* blood varies from 2.70% to 4.79% and that of *Octopus vulgaris* blood from 9.40% to 11.40%. Stewart *et al.* (1952) mentioned that the range in total protein concentration in the blood of *Cryptochiton stelleri* was from 1% to 3%.

Woods *et al.* (1958) carried out electrophoretic experiments with blood from 6 to 12 individuals belonging to each of 18 species of invertebrates, including *Loligo pealei* and *Ostrea virginica,* and stated (p. 519) that "within a given species differences in sex, size, or stages of the molt cycle produced no significant difference in the electrophoretic patterns."

Horn and Kerr (1963) have presented the most extensive data on the serum protein and copper (and by inference the hemocyanin) concentrations in the blood of *Callinectes sapidus,* the blue crab. They found a 10-fold variation in the total serum protein concentration and an 18-fold range in the serum copper concentration. There was no relationship of either serum protein or copper concentration to the size of the crab, but it was found that female crabs had significantly higher serum protein and serum copper concentrations. No similar data for any molluscs are available.

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In this paper I will document the low average concentrations and the rather unexpected variability in the amount of hemocyanin found in the blood of four species of *Haliotis*, including data from a total of 172 individuals.

MATERIALS AND METHODS

Collection of animals

Abalones of the species *Haliotis fulgens, H. corrugata, H. rufescens* and *H. cracherodii* were collected at intervals during the period from 1960 to 1962, mostly from the area around La Jolla, California. The depth from which they were taken varied from the intertidal region down to about 60 feet. Most of these animals were bled on the day that they were collected, while some were kept in aquarium tanks provided with running sea water for periods up to nearly two years before being bled; food (*Macrocystis pyrifera*) was provided during this period.

The numbers of animals included in this study were: *H. fulgens*, 107; *H. corrugata*, 26; *H. rufescens*, 32; *H. cracherodii*, 7.

Bleeding of animals

The abalones were removed from the water, quickly shaken to empty water from the branchial cavity, the shell dried with a towel and each animal weighed. The foot was then wiped with a towel, and a short incision, about 2 cm. deep, was made in the midline of the foot. The blood which welled into the cut from the large pedal sinuses was sucked up quickly with a large pipette, centrifuged to remove the few blood cells (the volume of cells was always less than 0.5%), and stored at 0° C. Blood from which the cells have been removed will be called plasma.

Gonad index

The gonad-hepatopancreas was cut at a point midway between the start and the distal end of the horn. The diameter of the whole organ was measured in two directions at right angles to each other, as was also the diameter of the inner hepatopancreas. The gonad index was calculated as follows:

$$\text{Gonad index} = \frac{\text{Avg. diam. of whole organ}}{\text{Avg. diam. of hepatopancreas}}$$

The sex of the animal was determined at the same time.

Total nitrogen and NPN

Total nitrogen was determined on 1-ml. aliquots of the plasma by the micro-Kjeldahl method (A.O.A.C., 1955).

Non-protein nitrogen was determined by the microKjeldahl method on protein-free filtrates prepared by the tungstic acid method of Folin and Wu (1919).

Protein was estimated by multiplying the protein nitrogen concentration by the conventional factor of 6.25. All determinations were done in duplicate.

Absorbance

Spectral absorption curves of the blood showed a broad, rounded maximum at 560 $m\mu$, as has been found for hemocyanins of other species. The absorbance, at 560 $m\mu$, of undiluted and thoroughly aerated plasma was determined as a measure of the hemocyanin concentration. The absorbance at 280 $m\mu$, a measure of the total protein concentration, was determined after dilution of 1 ml. of the plasma to 25 ml. with 0.5 N NaCl solution. Dilution with distilled water caused precipitation of the hemocyanin.

Copper

Aliquots of plasma (usually 1.0 ml.) were digested with 5 ml. of a mixture of 100 ml. of 70% perchloric acid and 400 ml. of concentrated nitric acid. Copper in the digests was determined according to method B given in Diehl and Smith (1958). In this procedure copper is reduced to the cuprous form, extracted as the 2,2′-biquinolate into isoamyl alcohol, and the absorbance of the colored complex measured at 546 $m\mu$. The addition of hydroquinone in a final concentration of 0.5% to prevent fading of the color, as recommended by Riley and Sinhaseni (1958), was found to give good color stability. All determinations were done in duplicate.

Electrophoresis

Horizontal paper electrophoresis was carried out at room temperature. Barbital buffer, pH 8.6, ionic strength 0.05, was used, and the voltage gradient was about 3 volts/cm. Plasma was applied to the strips without prior treatment; 10, 20, or 30 microliters were applied to each 3-cm.-wide strip. After the separation, the strips were stained with bromphenol blue.

RESULTS

Of the various measurements made on the plasma samples, the one for which most data are available is the concentration of total organic nitrogen. This varied

TABLE I

Average and range of total nitrogen and of hemocyanin in 4 species of abalones. Nitrogen as mg./100 ml., protein as gm./100 ml.

	H. fulgens	*H. corrugata*	*H. cracherodii*	*H. rufescens*
No. of animals	107	26	7	32
Total N				
Median	129.0	67.4	91.3	92.2
Low	33.0	28.8	60.5	47.1
High	358.0	275.5	342.5	214.5
Hemocyanin N, Median	86.5	23.8	60.1	—
Hemocyanin				
Median	0.54	0.15	0.38	—
Low	0.030	0.0017	0.210	—
High	1.89	1.53	2.03	—
Maximum variation	63-fold	900-fold	10-fold	—

from 33.9 to 358.0 mg./100 ml. for *H. fulgens*. Wide ranges of variation were also found for the other species (Table I). It will be shown first that the hemocyanin concentration is directly related to the total nitrogen concentration. Then the total nitrogen concentration, as a measure of the hemocyanin concentration, will be compared to the environmental and physiological parameters which have been measured. The tabulated raw data have been reported by Pilson (1963).

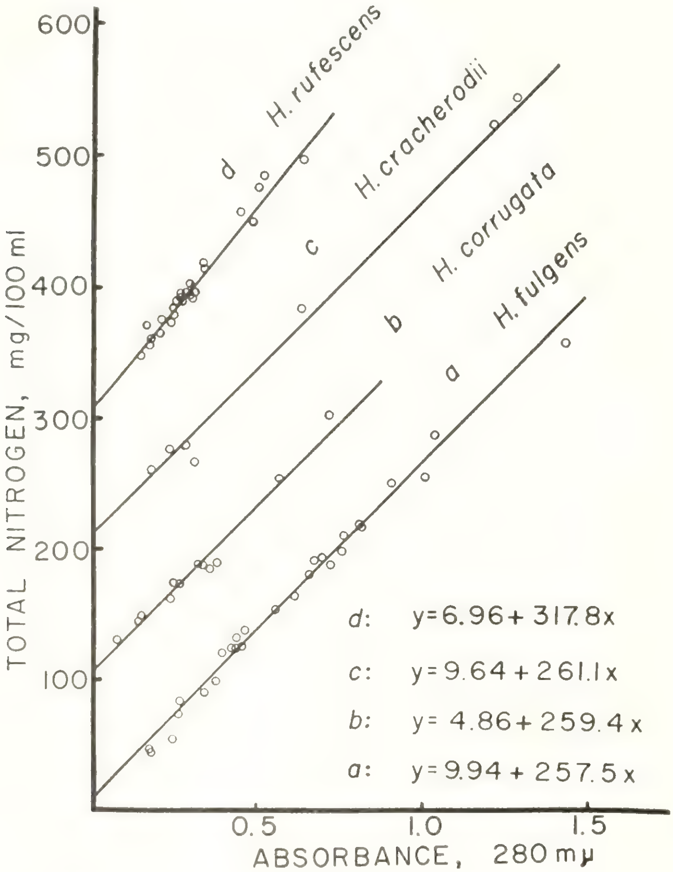

FIGURE 1. Total organic nitrogen concentration in plasma from 4 species of *Haliotis* *vs.* the absorbance at 280 mµ after dilution of 1 ml. of plasma to 25 ml. with 0.5 N NaCl solution. The plotted points for each succeeding species are displaced upward by 100 units.

Non-protein nitrogen

The median NPN found for *H. fulgens* (14 determinations) was 8.8 mg./100 ml. If the plasma does not contain appreciable quantities of non-protein substances absorbing in the region of 280 mµ, then the NPN can be estimated also from the relation of total nitrogen to the absorbance at 280 mµ. Figure 1(a) shows this relationship for the 27 samples for which these data were available. This and all subsequent regression lines have been calculated by the method of least squares. The y-intercept at 9.9 is satisfactorily close to the determined median value of 8.8 mg. N/100 ml. There is very little scatter about this line, indicating that large variations in the non-protein nitrogen fraction are unlikely.

Figure 1 (b and c) shows the same plots for *H. cracherodii* and for *H. corrugata*. The y-intercept for *H. cracherodii* is 9.6 mg. N/100 ml. and this is the only available estimate for NPN for this species. The y-intercept for *H. corrugata* is 4.9, close to the median value of 5.0 mg. N/100 ml. obtained by direct determination on three samples. Figure 1(d) shows the same relationship for *H. rufescens*, and the estimated average value for the NPN is 7.0 mg. N/100 ml.

These values for NPN are minimal values, for any non-protein substance in the plasma which absorbs at 280 mμ will tend to shift the curve to the right, thus depressing the value at the y-intercept.

The slopes of the regression lines are similar for the first three species, but that for *H. rufescens* is about 23% greater, indicating that the plasma proteins of *H. rufescens* may contain less of the amino acids which absorb at 280 mμ than do those of the other species.

Non-hemocyanin protein

The degree to which the variation in total nitrogen in the plasma is due to non-hemocyanin protein was estimated by paper electrophoresis, the relation of total nitrogen to the absorbance at 560 mμ, and by the relation of the total nitrogen to the copper concentration.

Paper electrophoresis patterns were obtained for samples of plasma from four individuals of *H. fulgens* and one from *H. corrugata*. Two faint bands were present on the strips prepared for all samples, and appeared to be approximately constant in intensity. A third band, of greater mobility than the others, with a mobility approximately that of human α-globulin (human serum was run at the same time as a control) was variable in intensity; by visual estimation this variation in intensity was thought to be approximately proportional to the blueness of the blood and to the total nitrogen content. This band was presumably due to hemocyanin.

All known hemocyanins have, in the oxygenated form, a broad absorption maximum in the region of 560 mμ. Plasma from *H. fulgens* had an absorption spectrum very similar to that of known hemocyanins, with a peak very close to 560 mμ. The absorbance in this region, then, can be taken as a measure of the amount of hemocyanin present. In Figure 2 the total nitrogen in the plasma is plotted against the absorbance at 560 mμ. The intercept of the regression line on the y-axis gives a minimal value for the non-hemocyanin nitrogen present. This is minimal because any other source of absorbance in the plasma will tend to shift the curve to the right, lowering the value for the y-intercept. The value for non-hemocyanin nitrogen is 24.0 mg./100 ml. for *H. fulgens*. The estimated values for the non-hemocyanin nitrogen in plasma from *H. corrugata* and *H. cracherodii* were 21.8 and 20.1 mg./100 ml., respectively. The plot of the values from *H. rufescens*, at the top of Figure 2, shows a different pattern. The 5 points enclosed in triangles appear to constitute a separate group. It seems that in this species there may be considerable variation in the concentration of the non-hemocyanin protein. No attempt was made to fit a regression line to these points.

Another way to estimate the non-hemocyanin protein is to assume that all the copper in the plasma is bound in the hemocyanin and then to examine the relationship between the total nitrogen present and the amount of copper in the plasma. In Figure 3 the total nitrogen in samples of plasma is plotted against the copper concentration. The regression line fitted to the points from *H. fulgens* has a y-intercept of 42.5. This is probably the best measure of the average amount of

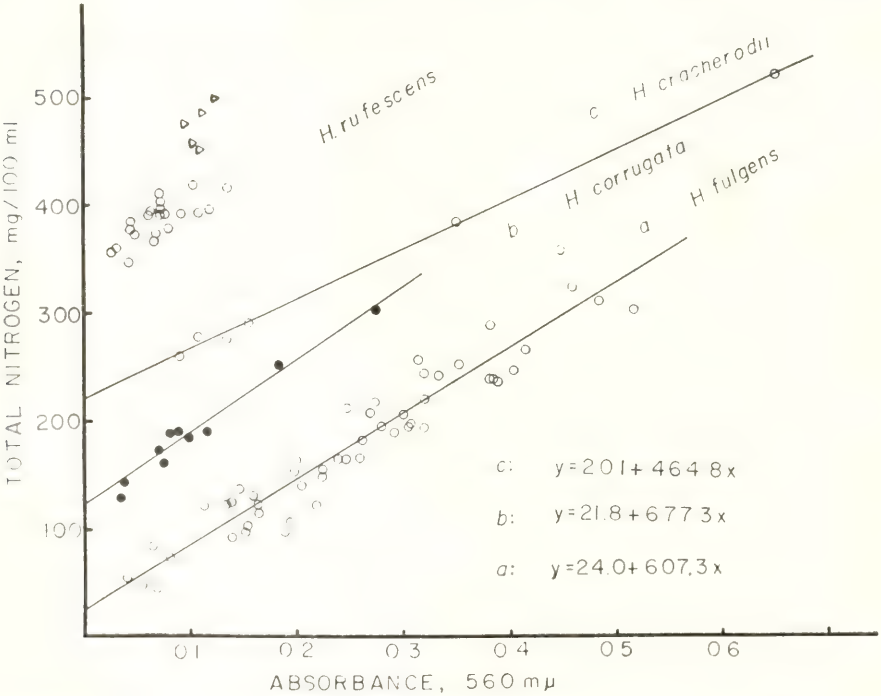

FIGURE 2. Total organic nitrogen concentration in plasma from 4 species of *Haliotis vs.* the absorbance at 560 mμ. The plotted points for each succeeding species are displaced upward by 100 units.

non-hemocyanin nitrogen present. It should be noted, however, that some samples of plasma had less than 42.5 mg. total nitrogen per 100 ml. Similar values for *H. corrugata* and for *H. cracherodii* were 43.6 and 31.2 mg./100 ml., respectively. The plotted data from *H. rufescens* again indicate that in this species the non-hemocyanin protein may be quite variable.

I corrected for the non-protein nitrogen and used the conventional conversion factor of 6.25 to calculate the following average amounts of non-hemocyanin protein present: *H. fulgens*, 204; *H. corrugata*, 242; and *H. cracherodii*, 135 mg./100 ml.

Hemocyanin concentration

Figure 4 shows the relationship between the absorbance at 560 mμ and the copper concentration of the plasma from *H. fulgens*, *H. corrugata* and *H. rufescens*. It is clear that, for the first two species named above, the absorbance at 560 mμ is well correlated with the copper concentration. This indicates that most of the copper is present in combination as hemocyanin. For both species the y-intercept is above 0, indicating that the plasmas absorbed or scattered light by an amount greater than could be attributed to the hemocyanin alone.

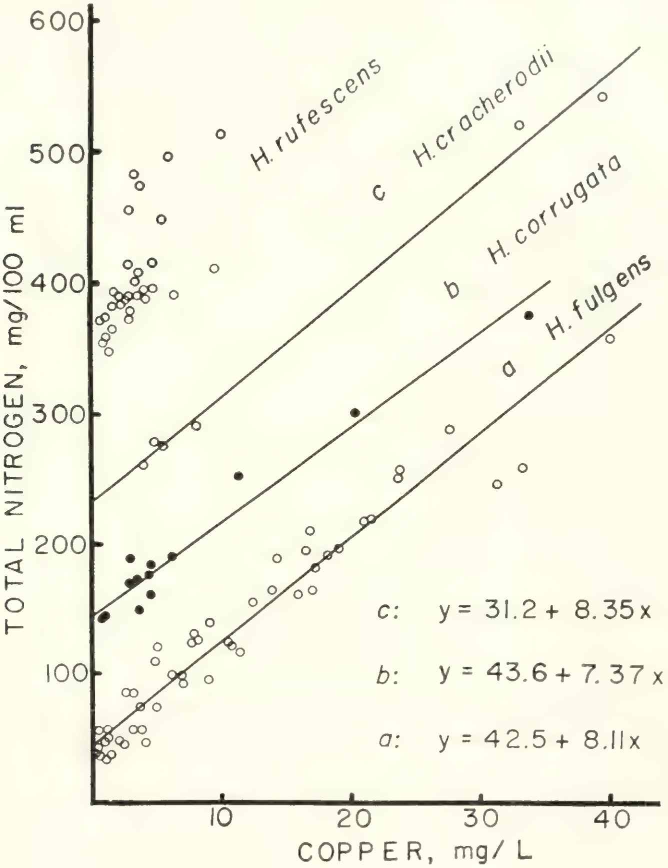

FIGURE 3. Total organic nitrogen concentration in plasma from 4 species of *Haliotis vs.* the total copper concentration. The plotted points for each succeeding species have been displaced upward by 100 units.

From the relationships that have been developed it seems that one can with some confidence use the total nitrogen, the absorbance at 560 mμ or at 280 mμ, or the copper concentration, to estimate the hemocyanin concentration in the plasma of all species investigated except *H. rufescens*.

The data which have been presented show that the concentration of hemocyanin in abalone blood is extremely variable. A frequency histogram, at 20-mg. intervals, of the total nitrogen in all 107 samples of plasma from *H. fulgens* is shown in Figure 5. Since the relationship shown in Figure 3 indicated that the non-hemocyanin nitrogen averaged about 40 mg./100 ml., the scale was shifted two intervals (40 mg.) to show an approximation to the frequency histogram for hemocyanin nitrogen. This distribution is not normal. It is relatively flat, and skewed to the right. The usual statistical measures are not entirely appropriate to this distribution; I have used the median and the range because I want to emphasize the physiological implications of the great range found.

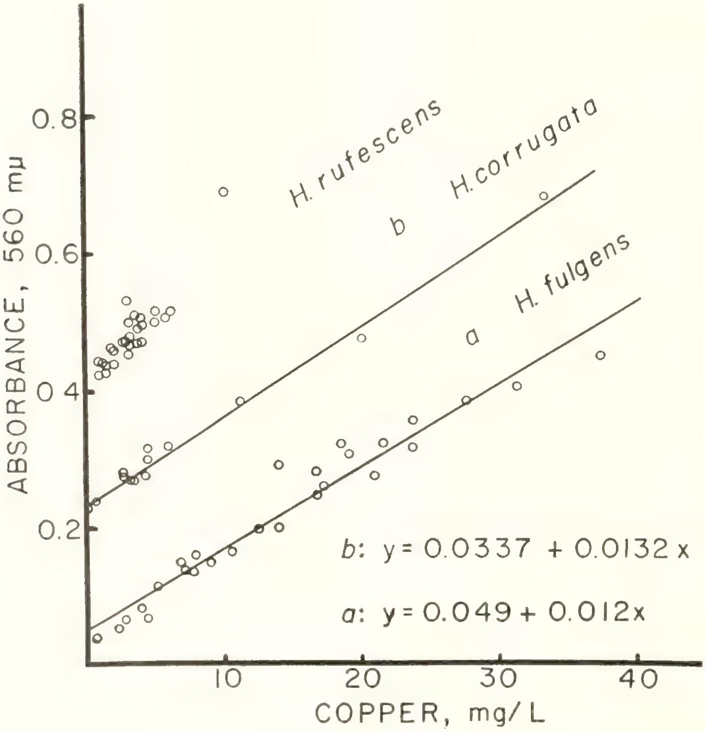

FIGURE 4. Absorbance at 560 mμ of plasma from 3 species of *Haliotis vs.* the total copper concentration. The points for each succeeding species have been displaced upward by 100 units.

Though Horn and Kerr (1963) did not point this out, it may be noted from their data that the statistical distribution of serum protein concentrations was different for male crabs, being more skewed toward the high end of the range, and having a much flatter distribution curve in comparison with the more nearly normal distribution of this value for female crabs.

The median value of total nitrogen for these 107 animals is 129.0 mg./100 ml., and the range is from 33.9 to 358.0 mg./100 ml. Subtracting the estimated value of 42.5 mg. of non-hemocyanin nitrogen gives a median value of 86.5 mg. of hemocyanin nitrogen per 100 ml. This may be multiplied by the factor 6.25 to give a median hemocyanin concentration of 0.54 gm./100 ml.

The range in hemocyanin concentration may be estimated from the total nitrogen figures by subtracting the estimate for non-hemocyanin nitrogen. This gives a range of 0 to 1.97 gm. of protein per 100 ml. However, no samples of plasma were encountered in which copper could not be detected; therefore, the *range* of hemocyanin concentration has been estimated from the copper concentrations. This varies from 0.59 to 37.2 mg. Cu/L. From the relationship given in Figure 3(a) it follows that these copper values correspond to a range of 4.78 to 302 mg. of hemocyanin nitrogen per 100 ml. The corresponding range for hemocyanin concentration is from 0.030 to 1.89 gm./100 ml. There is, then, in these 107 samples of plasma from *H. fulgens*, a 63-fold variation in the concentration of hemocyanin present.

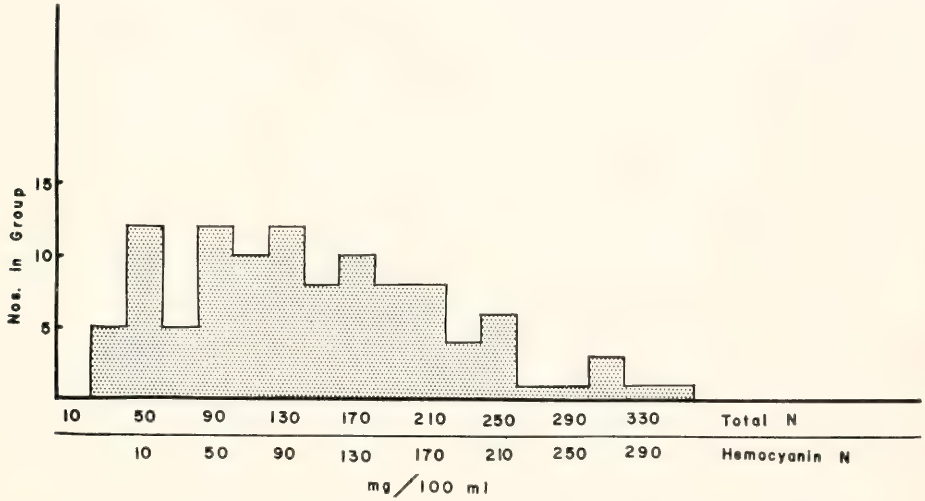

FIGURE 5. Frequency histogram of total organic nitrogen concentration in plasma from *H. fulgens*. The offset scale gives an approximation to the frequency histogram of hemocyanin nitrogen concentration.

These and similar calculations for the other species are summarized in Table I. No attempt was made to calculate average hemocyanin values for *H. rufescens*.

Median hemocyanin concentrations were 0.15 and 0.38 gm./100 ml. for *H. corrugata* and *H. cracherodii*, respectively, and the maximum variation found was 90-fold and 10-fold, respectively.

Weight and total nitrogen

Figure 6 shows the concentration of total plasma nitrogen in *H. fulgens* plotted against the weight of the animal. It is clear that the total plasma nitrogen concentration (or, by inference, hemocyanin concentration) is not related to the size of the animal. Probably it is not related to the age of the animal either.

Similar graphs for the other three species are equally unrewarding, and are not illustrated here.

Gonad index and total nitrogen

It might reasonably be supposed that the reproductive activities of the abalone, involving the synthesis of large amounts of gonad tissue and of eggs or sperm, might affect the hemocyanin or total nitrogen concentration of the plasma. Figure 7 shows the total nitrogen concentration in the plasma from *H. fulgens* plotted against the gonad index. It would appear that the total nitrogen of the plasma is unrelated to the reproductive state of the animal, as judged from the gonad index. Similar graphs for the other species show an equal lack of relationship.

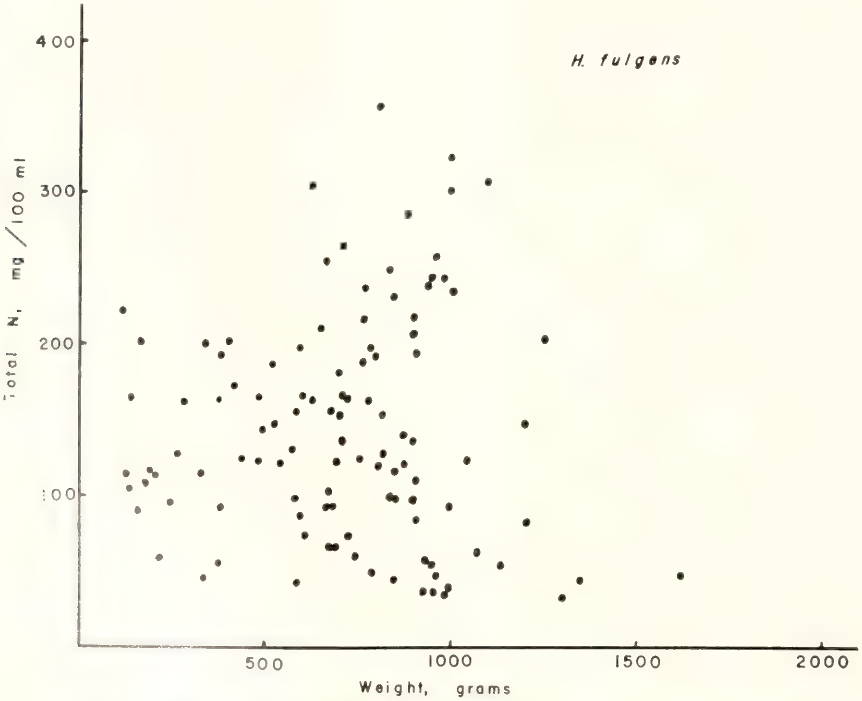

FIGURE 6. [illegible] nitrogen concentration in plasma from *H. fulgens* *vs.* the total weight of the animal.

There was no significant difference in the total nitrogen concentration of the plasma between male and female specimens of *H. fulgens.*

Nutritional state and total nitrogen

Five individuals of the species *H. corrugata* were collected from a depth of 35 feet off Point Loma in an area from which the kelp had been absent for at least a year. These animals all appeared shrunken and the foot muscle was weak and flabby; the gonads were undeveloped. Another group of five individuals, collected also from a depth of 35 feet only 200 yards away, had been living in an area where kelp was abundantly available. These were firm, healthy in appearance and with well developed gonads. The average total nitrogen content of the plasma from the starved animals was 62.1 mg./100 ml., and that from the well fed animals was 47.5 mg./100 ml. This difference was not statistically significant. From this admittedly small sample, it appears that hemocyanin concentration is not correlated with nutritional state. The great majority, and perhaps all, of the other animals examined were healthy and firm in appearance.

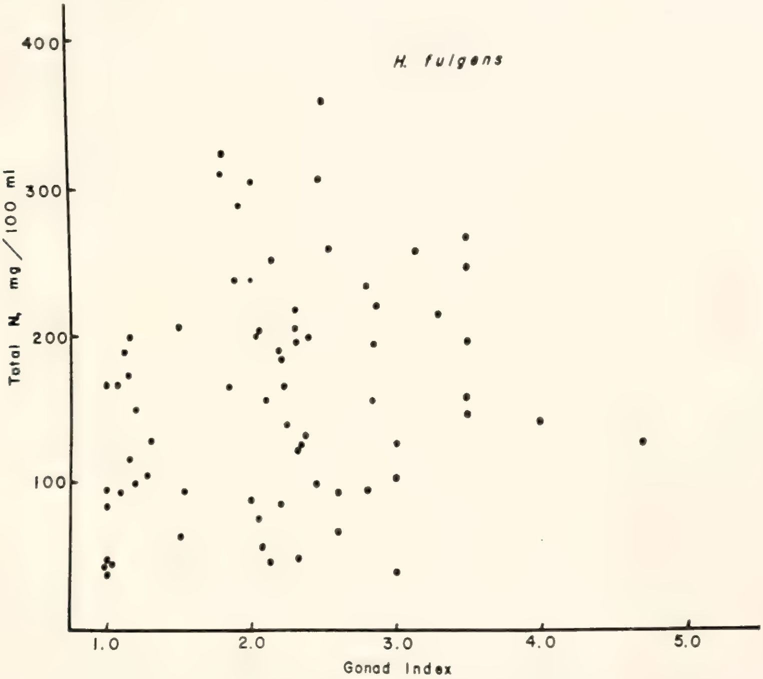

FIGURE 7. Total organic nitrogen concentration in plasma from *H. fulgens vs.* the gonad index.

Depth and season

Within the species *H. fulgens* there appeared to be no relationship between the concentration of total nitrogen in the plasma and the depth at which the animals had been collected.

Thinking that the warming of the water during the summer months might put sufficient stress on the abalones to cause an increased synthesis of hemocyanin, I plotted in Figure 8 the mean total nitrogen in various collections against the time of year. There is no obvious seasonal trend demonstrated in this figure.

Copper and hemocyanin

If it is assumed that hemocyanin from *H. fulgens* contains two atoms of copper for each molecule of oxygen bound, as is known to be the case for other species, it is possible to calculate, from the relationship shown by Figure 3, the minimum combining weight of the hemocyanin. For *H. fulgens* this is 10,300 gm. of nitrogen. Multiplying by the conventional factor of 6.25 yields a minimum weight of 64,400 gm. of protein. Comparable figures obtained by direct analysis of the hemocyanin from other molluscs are 52,800 for *Helix pomatia,* 51,800 for *Busycon canaliculatum,* and 50,800 for *Octopus vulgaris* (calculated from data in Prosser and Brown, 1961).

Severy (1923) reported the copper content of each of five whole individuals of *H. cracherodii* to be 0.8 mg./kg. of wet tissue. Marks (1938) reported the following whole-body copper concentrations in three species of abalone: *H. fulgens,* 2.5 to 10 mg./kg. (11 samples); *H. cracherodii,* 1 to 13 mg./kg. (6 samples); *H. rufescens,* 1 to 2 mg./kg. (3 samples). The average blood volume, determined by dilution of inulin, for three individuals of *H. fulgens* was 41% (Pilson, 1963). Thus, in this species the blood alone would contribute from 0.24 to 15.3 mg. of Cu per kg. of wet tissue. The data reported by Marks are more or less in accord with those reported herein, and indicate that, at least in the case of those animals with high concentrations of hemocyanin in the blood, the major part of the total body store of copper is probably present in the circulating hemocyanin.

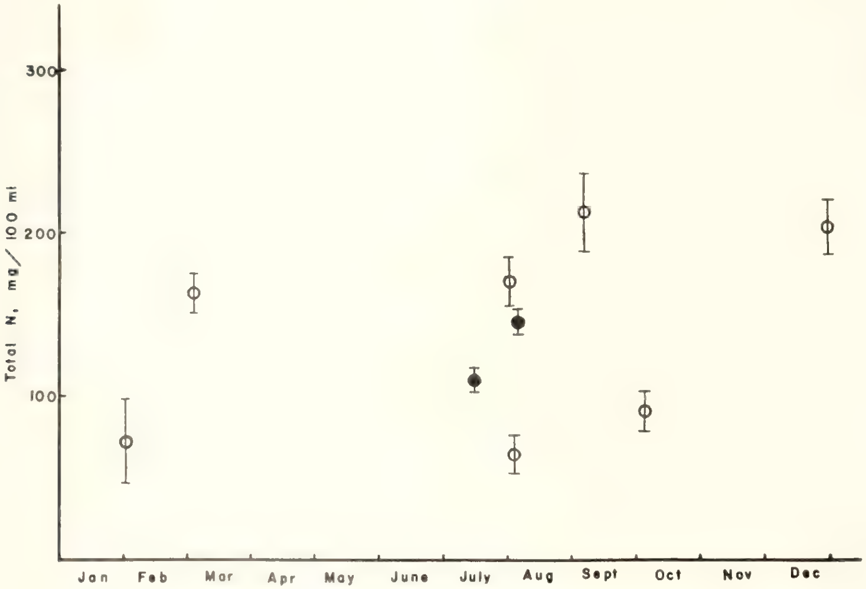

FIGURE 8. Average total organic nitrogen concentration in various groups of *H. fulgens vs.* the time of year when the group was collected. Open circles identify groups bled shortly after collection; closed circles identify groups maintained and fed in aquaria for 7 to 24 months. The limits given are the standard errors of the means.

DISCUSSION

The significance of this great variation in the concentration of hemocyanin in the blood of *Haliotis* to the physiology of the animal is not known. It is a common view that hemocyanin acts as a respiratory oxygen-carrier, and the known chemistry of this protein is not in conflict with this idea. Certainly hemocyanin in all known species can combine reversibly with oxygen. However, if some animals can survive with a certain concentration of hemocyanin in their blood why should other individuals of the same species have a concentration 60 times greater? It seems that such an enormous variation in the concentrations present is not compatible with any physiological function which has been suggested so far.

That hemocyanin in *Haliotis* might be a sort of evolutionary relic, in the process of disappearing, seems unlikely, for the variability was found in all four species investigated, although their ecologic distributions are different (Cox, 1962), and a range of hemocyanin concentration almost as great was also found in the blue crab (Horn and Kerr, 1963).

This work was submitted in partial fulfillment of the requirements for the Ph.D., University of California. I am grateful to Professor Denis L. Fox and Professor A. Baird Hastings for a critical reading of the manuscript. Financial support was received from the Quebec Agricultural Research Council and from the Ford Foundation during the tenure of a Sverdrup Fellowship. Dr. P. Taylor, Dr. R. Clutter, D. Leighton, and many others helped in the collection of specimens.

SUMMARY

1. The concentration of non-protein nitrogen in plasma from four species of *Haliotis* was fairly constant with average values estimated as follows: *H. fulgens*, 9.9; *H. cracherodii*, 9.6; *H. corrugata*, 4.9 and *H. rufescens*, 7.0 mg./100 ml.

2. The concentration of non-hemocyanin protein in plasma was fairly constant, and quite low, in three of the species studied. Average values, expressed as gm./100 ml., were: *H. fulgens*, 0.20; *H. cracherodii*, 0.14; *H. corrugata*, 0.24. Plasma from *H. rufescens* contained low but variable quantities of non-hemocyanin protein.

3. Hemocyanin was present in abalone plasma in greatly varying concentrations. The median hemocyanin concentration in the plasma of 107 individuals of the species *H. fulgens* was 0.54 gm./100 ml., and the range was 0.03 to 1.89 gm./100 ml. This is a 63-fold variation in concentration.

4. Plasma from *H. corrugata*, *H. cracherodii* and *H. rufescens* also had varying concentrations of hemocyanin. Plasma from 26 individuals of *H. corrugata* had a median concentration of 0.15 gm./100 ml., and seven individuals of *H. cracherodii* had a median hemocyanin concentration of 0.38 gm./100 ml. The corresponding ranges were 0.0017 to 1.53, and 0.210 to 2.03 gm./100 ml., respectively, giving a 900-fold and a 10-fold range between the highest and lowest samples. No similar information is available for other species of molluscs.

5. The concentration of hemocyanin was unrelated to the animal's weight, sex, reproductive activity, as judged by the gonad index, nutritional state, the depth in the water at which the animal had been collected, or the season of the year.

6. This enormous range in concentration of hemocyanin in the blood appears to be incompatible with any physiological function which has so far been suggested.

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PIGMENT MIGRATION AND LIGHT-ADAPTATION IN THE EYE OF THE MOTH, GALLERIA MELLONELLA[1]

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With few exceptions, the compound eyes of arthropods fall into two groups—those with relatively short rhabdoms and pronounced migrations of accessory shielding pigment, and those with rhabdoms extending the length of the retinulae and little or no movement of pigment (Exner, 1891). The former occur in nocturnal animals or species which are found where there is low illumination; the second type is characteristic of diurnal forms which are active where there is ample light.

Because of this ecological correlation it has been widely assumed since the time of Exner that migratory pigment is an adaptation for vision in dim light; however, physiological evidence in support of this view has been provided only recently. The eyes of moths dark-adapt in two steps, but the second, slower phase does not, as in the human eye, represent a second population of receptors with lower thresholds. The increase in sensitivity of the eye during this slow phase runs parallel with the migration of shielding pigment as it moves distally to a compact mass between the crystalline cones (Bernhard and Ottoson, 1960a, 1960b, 1964; Bernhard, Höglund and Ottoson, 1963). Eyes which have no migratory pigment lack the second component in the dark-adaptation curve (Bernhard and Ottoson, 1960a; Bernhard, Höglund and Ottoson, 1963; Goldsmith, 1963), and in the fully dark-adapted state they are not as sensitive as eyes in which the pigment sleeves have withdrawn distally (Bernhard, Höglund and Ottoson, 1963). Compound eyes with short rhabdoms and migratory pigment can appropriately be called *scotopic* eyes; those with no migratory pigment, *photopic*.[3]

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[2] Supported in part through the N.S.F. Undergraduate Science Education Program at Yale University.

[3] A brief consideration of terminology is necessary here. The traditional names for these types of compound eye are superposition and apposition, and were introduced by Exner (1891). These terms refer to supposed differences in refractive properties and mode of image formation which Exner described in great detail but which subsequently have been shown not to be general properties of the two groups (Kuiper, 1962; Goldsmith, 1964). The *raison d'être* of these names is thus rooted in an erroneous analysis of the optics of compound eyes, and the only argument for perpetuating them is that they have existed now for almost three-quarters of a century. We feel they should be replaced by terms that relate to the known properties of the eyes and that are established in the vocabulary of visual physiology. By scotopic eye we thus mean one which is of the morphological type which was supposed by Exner to form superposition images and which is adapted (in an evolutionary sense) for high sensitivity in dim light. Obviously the basis for this adaptation—migratory shielding pigment—is different from scotopic vision mediated by a rod-rich vertebrate retina. There remains a relatively small number of compound eyes with the photopic (apposition) morphology but which possess migratory pigment. What their proper physiological place may be must await experimental analysis.

In many Lepidoptera light controls migrations, either by direct action on the pigment cells or indirectly through the sense cells; the most recent work indicates that pigment migrations can be observed in isolated retinas and that connection with the optic ganglion or the circulation is not necessary (Tuurala, 1954). In such moths, cold or narcosis causes the pigment to move to the light-adapted position (Day, 1941), suggesting that the process which holds the pigment in the dark-adapted state requires metabolic energy and that possibly the effect of light is to inhibit this process.

The first part of this paper is a quantitative study of the light energy and time necessary to effect pigment migration in the scotopic compound eye of the wax moth, *Galleria mellonella.* The second part deals with the rate of light adaptation and how it is influenced by the presence of migratory pigment.

I. The Control of Pigment Migration by Light

Methods

Intact moths (*Galleria mellonella*) were placed on their backs and secured to a small cork platform with soft wax ("Tackiwax"). After a minimum of one hour dark-adaptation the animals were exposed to an adapting light for varying lengths of time.

The adapting light was obtained with a high-pressure xenon arc and a Bausch and Lomb grating monochromator, and consisted of a narrow band of wave-lengths (10 mμ half band width) centered at 500 mμ. An image of the diffraction grating was focused on the animals' heads. Intensity was controlled with a pair of calibrated optical wedges. Energy flux was measured with an Eppley bismuth-silver thermopile of known sensitivity and a Keithley No. 149 millimicrovoltmeter.

After a specified exposure the animals were decapitated under dim red light, the heads bisected, and the halves placed in Duboscq-Brasil fixative in the dark for 12–16 hours. The tissue was dehydrated and imbedded in paraffin by standard techniques. Sections 10 μ thick were cut parallel to the ommatidial axes, cleared of paraffin, and examined without staining with a phase contrast microscope to assess the extent of pigment migration. Some sections were depigmented with Grenacher's solution and stained with Harris's haematoxylin to aid in orientation.

Pigment position was measured as the distance between the distal end of the crystalline cone and the proximal border of the secondary pigment (see Fig. 1); but in order to compare eyes of different sizes, this figure was expressed as a fraction of the distance from the cone to the basement membrane. For ease in understanding, this index has been expressed in Figures 2 and 3 as a percentage of the total possible migration observed in eyes exposed to direct sunlight.

Experiments were performed in midafternoon during the months of June, July, and August, with animals reared in the laboratory by the method of Dutky, Thompson and Cantwell (1962).

Results

Figure 1 shows the positions of the pigment in dark- and light-adapted eyes. The nuclei of the retinular cells, which are located in the distal processes and are not readily visible in Figure 1, also migrate proximally in the light.

The extent of pigment migration after 3 and 27 minutes irradiation is shown in Figure 2 as a function of incident energy. Not only does the pigment move farther the higher the energy, but for each flux the pigment migrates more in 27 minutes than in three.

These conclusions are amplified by Figure 3, which shows the time course of migration for two intensities of light. In each case migration starts immediately at a relatively high rate, then slows as the pigment reaches a steady-state position characteristic of the incident energy. The time required to attain the final steady-state position is longer the farther the pigment moves and is of the order of half an hour for the nearly complete migration produced by 5×10^3 ergs sec.^{-1} cm.^{-2} at 500 mμ.

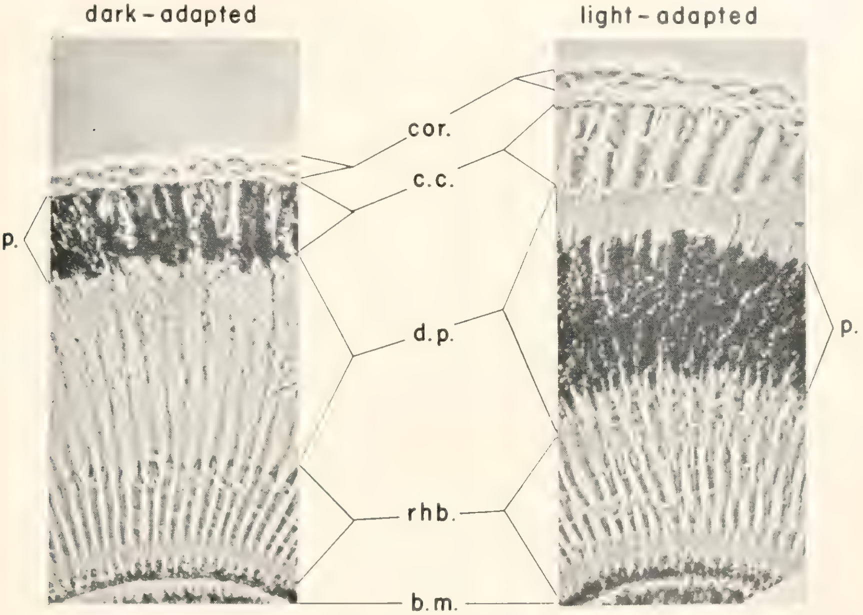

FIGURE 1. Sections of *Galleria* eyes cut approximately parallel to the long axes of the retinulae and showing the positions of the migratory pigment in the fully light- and dark-adapted states. The nuclei of the retinular cells, which are in the distal processes but are not readily visible without staining, also migrate distally in the dark. Sections were unstained and were photographed with phase-contrast optics. As a scale reference, the cystalline cones are about 45 μ in length. *Cor.*, cornea; *c.c.*, crystalline cone; *d.p.*, distal processes of the retinular cells; *rhb.*, rhabdoms; *b.m.*, basement membrane; *p.*, migratory pigment of the secondary pigment cells.

Discussion

Eyes exposed to the same combinations of intensity and time frequently show wide variation in pigment position; consequently a great many sections must be prepared to achieve a high precision of measurement, and this has discouraged us from measuring an action spectrum by this histological procedure.

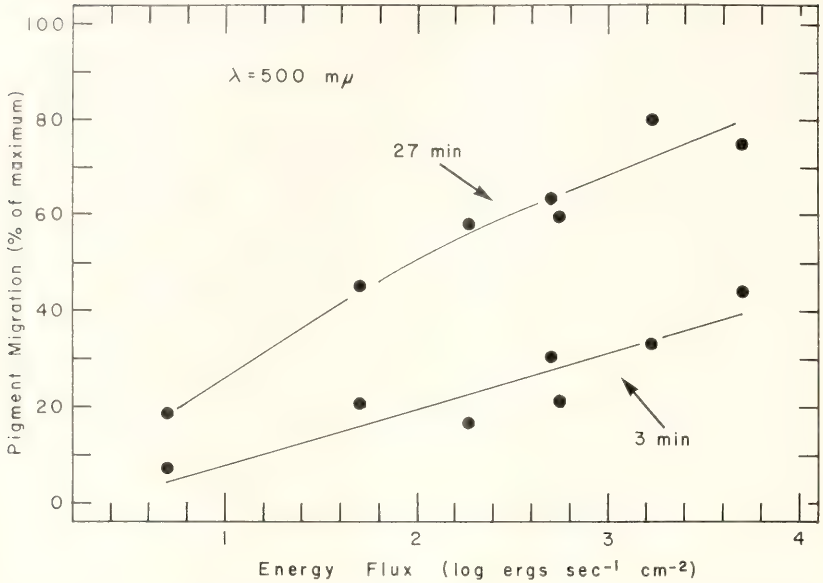

Figure 2. Proximal pigment movement as a function of energy for two different times of exposure, as determined from histological sections similar to those in Figure 1. Each point represents the average of about a dozen measurements on several sections from at least two eyes, each from a different moth. Energy flux was measured with a thermopile at the surface of the cornea. Wave-length was 500 mμ.

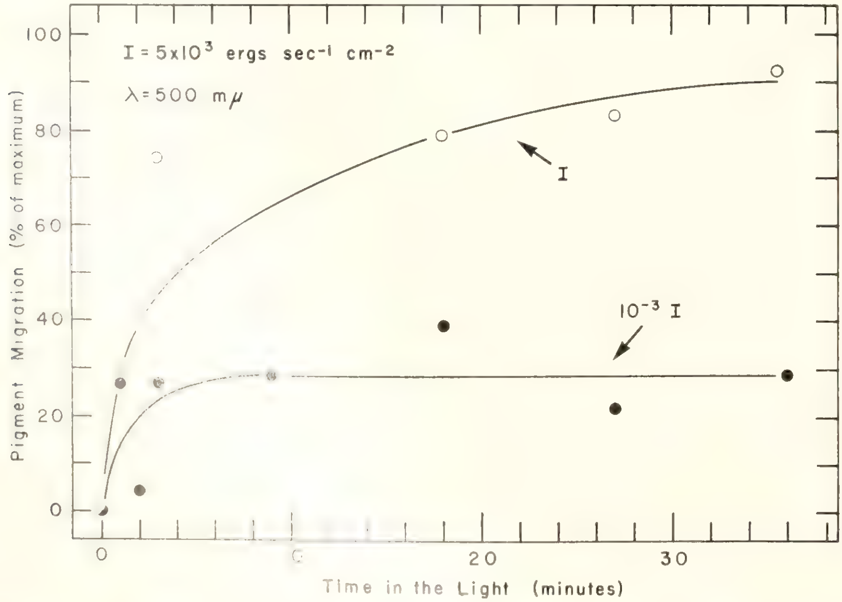

Figure 3. Proximal pigment movement as a function of time in the light. Details as in Figure 2.

The controlling effect of light is not all-or-none, for the extent of migration is a function of intensity. This is consistent with the thought that the rate of a chemical reaction necessary for the pigment to be held in the distal (dark) position is slowed by light, but a more precise kinetic model cannot be tested critically on the basis of the experimental observations presently available.

The finding that the pigment assumes intermediate positions in the presence of dim lights is in agreement with recent work of Bernhard and Ottoson (1964) who observed in *Cerapteryx graminis* a partial retraction of pigment towards the dark position when the intensity of a steady adapting light was decreased but not extinguished completely. Our observations on the time course of pigment movement are in semiquantitative agreement with Höglund (1963a), who reports that the large area of glow characteristic of dark-adapted eyes of various nocturnal Lepidoptera becomes minute when the pigment occupies a position intermediate between the distal and proximal extremes; and further, that glow requires 10 minutes or more to disappear when the eye is exposed to light. In a later paper Höglund (1963b) shows a case in which glow has vanished in less than 5 minutes. Both of these times are in reasonable agreement with our results, considering that different species were studied and that there is no precise information on Höglund's adapting energies or the relation between pigment position and the point at which glow vanishes.

II. The Rate of Light-Adaptation

Bernhard and Ottoson (1960a, 1960b, 1964) and Bernhard, Höglund and Ottoson (1963) have shown that dark-adaptation of moths has two components which, under some conditions, are completely separated in time. The second phase is caused by the distal migration of the shielding pigment and produces a fall in threshold of the eye of 1–3 log units. It does not indicate an intrinsic change in sensitivity of the retinular cells, but rather an increased probability that light crossing the cornea will reach the receptors (Höglund, 1963b).

The correlation between sensitivity change and pigment position during dark adaptation of scotopic eyes prompted us to examine the corresponding relationship during light-adaptation. A photopic compound eye lacks both pronounced pigment migration and the slow phase of dark adaptation (Bernhard and Ottoson, 1960a, 1960b; Bernhard, Höglund and Ottoson, 1963). Moreover, the rate of light-adaptation—measured as the change in increment threshold in the presence of the adapting light—is very rapid; a steady value of sensitivity is achieved within seconds after turning on the adapting light (Goldsmith, 1963). On the other hand, in a scotopic eye where pigment moves for minutes after exposing a dark-adapted eye to the light, one might, at least on first thought, suppose that light-adaptation should be correspondingly extended in time and increased in extent. The following experiments are in agreement with Höglund (1963b) in showing that this expectation is—for good reason—usually not fulfilled, but they indicate a greater variety in the shapes of light-adaptation curves than is suggested by his short but excellent paper.

Light adaptation curves of scotopic compound eyes have a multiple origin. There is a rapid fall in sensitivity as the receptors adjust their thresholds to the adapting light, but with the inward migration of pigment there are two additional effects which are opposed to one another. The test light reaching the receptors is attenuated, and this leads to a further *fall* in sensitivity of the *eye;* however, the adapting light is also decreased by the same pigment screen, and this permits the *receptors* to *gain* sensitivity. This was shown (Höglund, 1963b) by conducting the light stimulus to the receptors through a glass fiber inserted below the pigment, but we have come to the same conclusion from different experimental evidence. Rapid light-adaptation in the presence of pigment migration therefore means that the effect of attenuation of the test flash by the shielding pigment is approximately balanced by dark-adaptation of the receptors.

Methods

Optical. The test and adapting lights were 6-v. tungsten filament microscope lamps placed in light-tight metal boxes fitted with camera shutters. For most experiments the light paths originated at right angles to each other; they were combined with a beam splitter and arrived at the cornea on the same optical path. In some experiments a second test light was arranged so as to stimulate the eye from another angle.

Intensity of the test light was controlled with a circular optical wedge, and the adapting light with neutral density filters. In the experiments described below, intensity of the adapting light was sufficiently great to move the pigment to the proximal position. Infrared was attenuated with 1.25 cm. of a 10% (w/v) copper sulfate solution. Duration of the test exposures was monitored with a photocell.

Recording. Animals were immobilized in soft wax at the focus of the light beams. A shield of aluminum foil prevented light from reaching parts of the animal other than the illuminated eye. The retinal action potential was recorded with silver:silver chloride electrodes which made contact with the animal through a Ringer-filled capillary with a tip diameter of about 20 μ placed beneath the cornea of the illuminated eye, and a saline-soaked wick on the dark side of the head. The Ringer was that of Ephrussi and Beadle (1936). The responses were recorded with a direct-coupled amplifier (Grass P-6) and photographed from the face of an oscilloscope.

[illegible] A series of responses to increasing intensities was recorded for the dark-adapted eye and whenever the animal was held for an extended period in a light-adapted state. The test flashes were 0.5 sec. The height of the sustained negativity was plotted as a function of log I. Response-energy curves for the light-adapted eye were parallel, or nearly so, to those obtained from the dark-adapted eye, as found in [illegible] by Bernhard and Ottoson (1960a). Their displacement on the energy axis gives the change in sensitivity of the eye.

During light- and dark-adaptation the eye was stimulated every ten or more seconds with a test flash of constant intensity. The shifting position of the response-energy curve, and thus the changing sensitivity of the eye, was determined by comparing the size of each potential with the response-energy curve for the dark-adapted eye (*cf.* Hartline and McDonald, 1947; Goldsmith, 1963). For the measurement of dark-adaptation, test flashes were chosen that could be presented to the completely dark-adapted eye at 10-second intervals without themselves producing light adaptation.

Adaptation measurements are usually presented in terms of threshold rather than sensitivity. The term [illegible] threshold is used below synonymously with

-log relative sensitivity. It corresponds to the log of the intensity required for a constant effect.

Twenty-three animals were studied.

Results and Discussion

Figure 4 shows that dark-adaptation of *Galleria mellonella* sometimes splits into two components. As shown by the more typical recovery curve in Figure 5, however, this is a species in which the two limbs of the curve are usually not distinct.

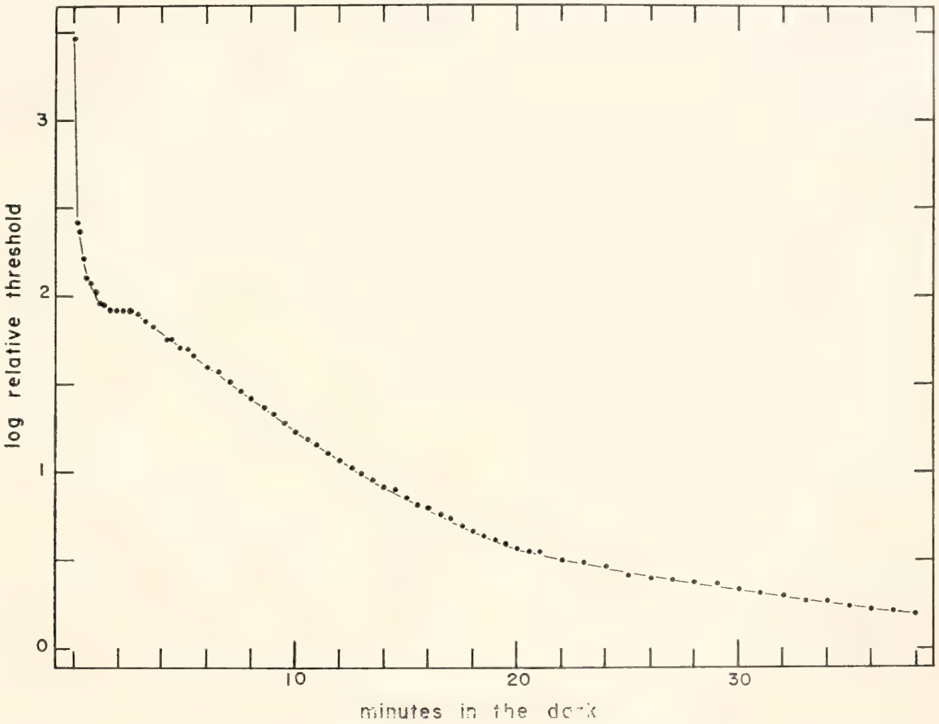

FIGURE 4. Dark-adaptation of the compound eye of *Galleria mellonella* in an animal showing a distinct latency before pigment migration started.

Overlap of pigment migration with physiological recovery has also been observed in other species (Bernhard, Höglund and Ottoson, 1963). It means that following the extinction of the adapting light the dark migration of pigment has commenced with little or no latency. The important point, however, is that dark-adaptation of *Galleria* is increased in extent and prolonged in time by the presence of migratory shielding pigment, just as in other species studied by the Swedish workers.

Not so light-adaptation. At least usually not; we shall consider exceptions in due course. Figure 6A (filled circles) shows the abrupt rise in threshold of an initially dark-adapted eye as a function of time after turning on a steady adapting light. Within seconds the sensitivity has reached its final value, even though the

Histological observations of pigment movement indicate that migration requires minutes for completion.

Figure 6A also shows that the wave form of the retinal action potential changes with time. The significance of this observation can be clarified by examining how the same eye responded when dark-adapted and stimulated by a series of flashes of increasing intensity (Fig. 6B). Ganglionic components are small and are most evident at low intensities and at "on"; the response is dominated by a graded potential which rises slowly and persists as long as the stimulus. With increasing intensity the rate of rise increases. At high intensities, instead of rising smoothly to a plateau there is an initial peak, about 100–200 msec. duration and graded with intensity, preceding the final steady value. This initial peak is evident in the response to the brightest light (log $I = 0$). Evidence of other kinds, including intracellular recording, suggests that both the initial peak and the plateau arise in the retinular cells (see Ruck, 1962 for review); for present purposes, however, it

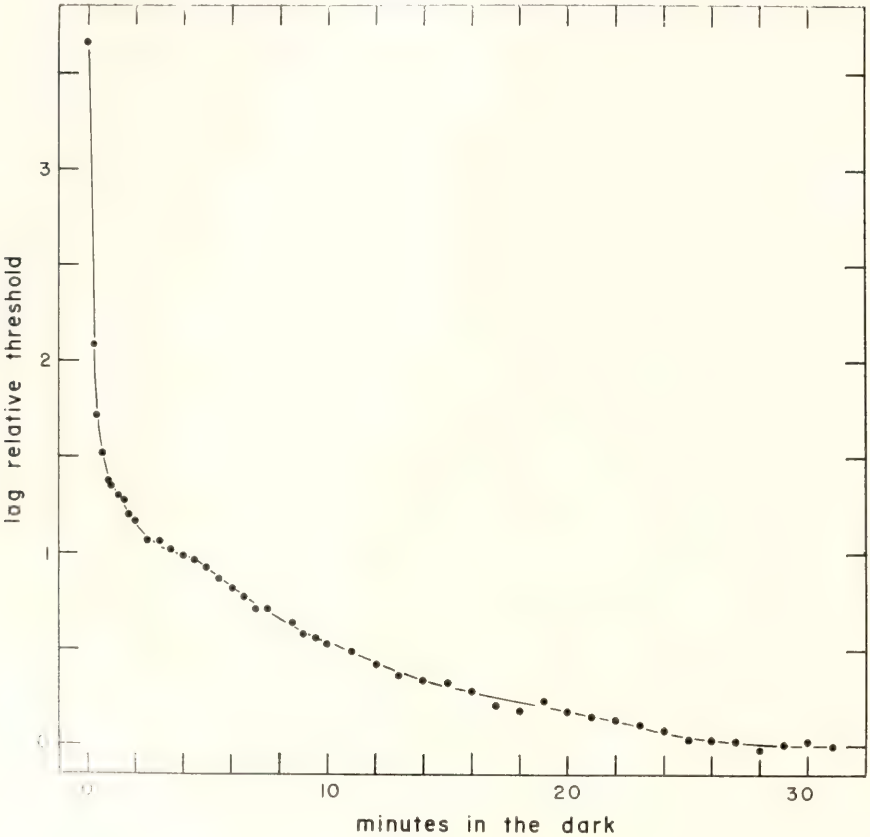

FIGURE [illegible]. A more typical dark-adaptation curve than Figure 4, showing that in this species [illegible] migration usually overlaps the earlier, faster components of recovery.

is sufficient to note that the initial peak is observed only when the cells are illuminated strongly.

The test flash used for the light-adaptation in Figure 6 was log $I = -1$, which produced a barely perceptible initial peak in the dark-adapted eye. On turning on the adapting light the response was decreased, but the initial peak became more prominent relative to the plateau (*cf.* responses at 0 and 24 sec.). The latter is the expected result of having more light—*i.e.*, both beams—incident on the receptors.

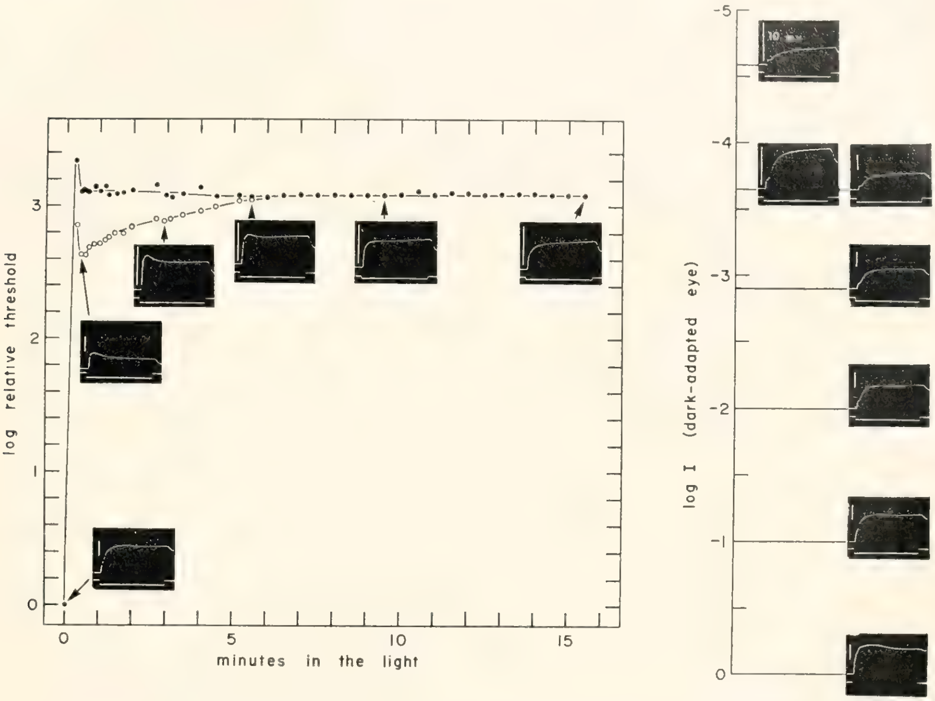

FIGURE 6. Light-adaptation of a compound eye of *Galleria*. Filled circles, based on the maintained plateau of the retinal action potential; open circles, based on the greatest excursion of the response. Several of the oscillograms from which the experimental points were calculated are included with the curve to show the progressive change in wave form of the retinal action potential during light adaptation. To the right of the graph is a series of responses to different intensities of stimulation recorded from the same animal while the eye was dark-adapted, just prior to irradiating with the adapting light. Test flashes were 0.5 sec. duration; a 10 mv. calibration mark is included with each trace.

The change in shape of the electrical response poses a problem: should one calculate sensitivity on the basis of the plateau or the highest part of the wave form? It is not obvious which is most important at the first synapse, so the issue has been avoided in Figure 6 by plotting both results. The filled circles are determined from the plateaus; the open circles are based on the greatest excursion of the response, which in the early minutes was the initial peak. By six minutes the peak had subsided, however, and the two light-adaptation curves meet. Even between 9.5 and 15 minutes, though, the form of the response continued to change; the height of the response remained constant, but its rate of rise decreased.

These progressive changes in the shape of the retinal action potential are characteristic of decreasing intensities of stimulation. They can be accounted for by a gradual diminution of the light reaching the receptors caused by the proximal migration of shielding pigment. The pigment is known to be moving during this time, even though the sensitivity of the eye is constant.

That attenuation of the test and adapting lights should produce equal and opposite effects on the sensitivity of the eye is a consequence of two factors: (a) equal absorption by the screening pigment, and (b) the applicability of Weber's law at the level of the retinal action potential. Insofar as the test and adapting beams are coaxial, for each position of pigment, absorption of the two lights will be the same. When the intensity of the test light reaching the receptors is decreased by the pigment screen to 0.1 of a previous value, clearly the energy in the flash will have to be increased ten-fold to maintain a constant response from the receptors; that is, sensitivity of the eye will have *fallen* one log unit. But if $\Delta I/I$ (ratio of incremental threshold to adapting intensity) is constant, at least over a significant range, the fall in intensity of the adapting light will produce an exactly equivalent *increase* in sensitivity of the receptors. Writing Weber's law,

$$\frac{\Delta I}{I} = \frac{I_t - I_a}{I_a} = k$$

where I_t and I_a are the intensities of the test and adapting lights, one can see that

$$I_t = I_a(k + 1)$$

$$\log I_t = \log I_a + \text{const.}$$

In other words, the curve log I_t *vs.* log I_a has a slope of unity (except close to threshold where the simple form of Weber's law used here must be modified), and a decrease of one log unit in I_a will produce an increase of one log unit in the sensitivity of the eye (Fig. 8).

The rapid rates of light adaptation as shown in Figure 6A are not always observed. Figure 7 compares the light adaptation of two other animals, one rapid, the other much slower. Both curves come within 0.2 log unit of the same final value and are based on the plateau of the retinal action potential. Other animals light-adapted at intermediate rates.

The problem is not why the time course of light-adaptation is usually rapid in scotopic eyes, but why it is relatively slow in some. A slow rate of light-adaptation means that for each position of pigment (a) the test flash is attenuated more than the adapting light, or (b) the decrease in energy of the adapting light fails to produce an equal dark-adaptation of the receptors. We shall consider each of these possibilities in turn.

What might be expected from unequal absorption of the test and adapting lights? After the initial rise in threshold observed during the first few seconds, further changes will depend on appropriately weighted averages of the optical

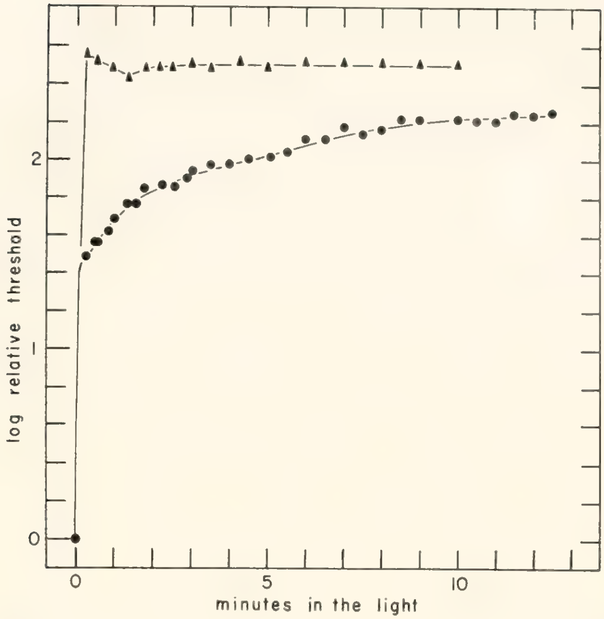

FIGURE 7. Light-adaptation of two eyes, illustrating the variation in rate which is observed in different animals. Both curves are based on the height of the plateau in the retinal action potential.

densities of the light paths in front of the receptors:

$$\text{log recovery} \propto \text{O.D. of adapting path}$$
$$\text{log rise in threshold} \propto \text{O.D. of test path}$$

Consequently,

$$\Delta \text{ log threshold} = (\text{O.D. test path}) - (\text{O.D. adapting path}).$$

This difference in optical densities will vary as a function of time until pigment migration is complete.

With coaxial beams it is not obvious how either light could be selectively screened, for both beams entered the eye along the same path. Nevertheless, in the hope of accentuating a selective absorption we have performed several experiments with one or the other of the beams impinging on the eye obliquely. When the test light strikes the pigment screen obliquely and the adapting light enters more or less axially, the O.D. of the test path will likely be greater than the O.D. of the adapting path, and Δ log threshold will continue to rise until the pigment has reached the proximal position. On the other hand, if the adapting light is oblique and the test light axial, the difference in O.D. will be negative and log threshold should fall somewhat. When the optical densities are equal (coaxial beams) there will be no further change in threshold irrespective of pigment position.

Of three experiments with an oblique test light, adaptation was very slow in two but essentially instantaneous in the third. In three experiments with an oblique adapting light the threshold rose to a maximum but did not subsequently decrease. In two of these animals the time course was rapid, but in the third it was slow. In these experiments there is therefore no clear demonstration of the effects that are expected as a result of differential absorption.

The most convincing experiments, however, are two in which light-adaptation was measured with a pair of test flashes presented alternately at intervals of several seconds, one oblique and a second coaxial with the adapting beam. These are shown in Figure 9. In each case the change in sensitivity to the axial test light was greater than to the oblique test light. This means that the populations of receptors responding to the two test lights were not identical and that the adapting light did not have an equal effect everywhere in the eye. In each experiment the time courses of adaptation were the same with the two test lights, and in one experiment they were very rapid. The difference in the shapes of the light-adaptation curves clearly depends on something other than the geometric arrangement of stimulating and adapting lights. Thus, under the conditions of these experiments there is no evidence for preferential absorption of either beam.

This brings us to the second possibility—that under some conditions there is an asymmetry in the physiological *effects* of decreased test and adapting lights. For example, attenuation of the adapting light might be more rapid than the ensuing equivalent recovery of the receptors. This could be observed if in some individuals resynthesis of visual pigment or some other physiological component of recovery were unable to keep pace with the proximal migration of screening pigment, either

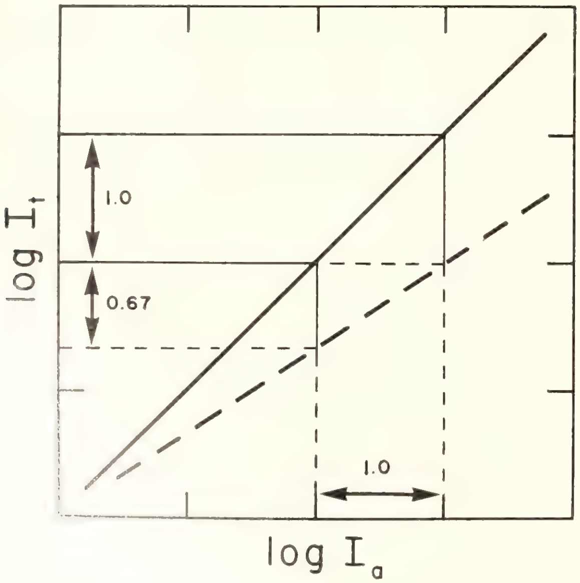

FIGURE 8. Solid curve: part of the increment threshold function for a photoreceptor which obeys Weber's law. A difference in the adapting intensity leads to an equal recovery of the receptor. Broken curve: part of the same function for a receptor in which a drop of adapting intensity of 1.0 log unit produces only a 0.67 log unit fall in the increment threshold. See the text for a discussion.

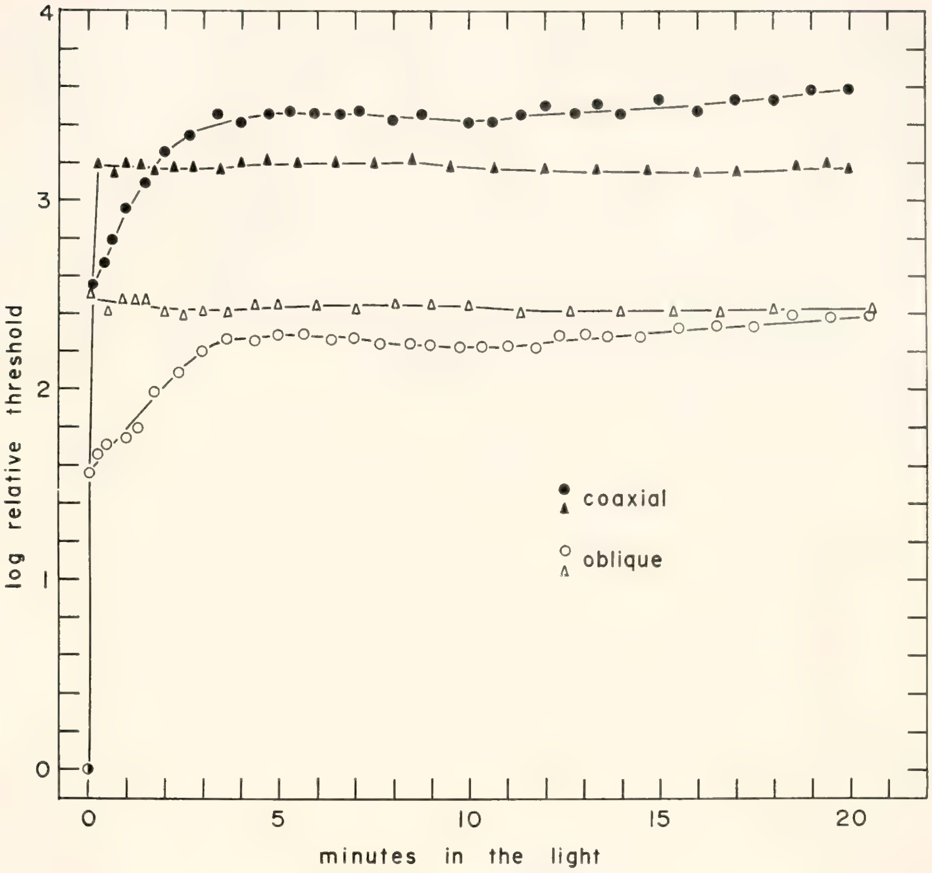

FIGURE 9. Pairs of light-adaptation curves for two animals (circles and triangles). Each pair of curves was measured during the same adapting exposure by stimulating the eye alternately with test flashes coaxial with the adapting beam (filled symbols) and obliquely (about 70°) to the adapting beam (open symbols). One animal (triangles) light-adapted much more rapidly than the other, irrespective of the angle between the test and adapting lights. All curves were based on the plateau in the retinal action potential.

because the former were unusually slow or the latter unusually rapid. If such were the case, *slow* light-adaptation would be observed in eyes with the *fastest* rates of pigment movement. Whether these rates vary sufficiently to account for the different rates of light-adaptation which have been observed is not known. Judging from the speed of dark-adaptation of photopic eyes, however, it seems unlikely that recovery of the receptors of scotopic eyes would lag behind movement of the pigment.

Alternatively, attenuation of the adapting light might not be followed by an equal dark-adaptation. If the increment threshold function, plotted as log I_{test} *vs.* log I_{adapt}, had a slope less than 1.0, partial dark-adaptation of the receptors caused by screening of the adapting light would not completely offset the rise in threshold caused by attenuation of the test light (Fig. 8). Measurements of this slope in seven animals gave values ranging from 1.0 to 0.67. The mean was 0.88 with a standard deviation of ±0.16, and the distribution was skewed toward lower values. In each case the measurements were made over approximately the same range of intensities. In the animal which provided the smallest value, attenuation of the test and adapting light 1 log unit by screening pigment would permit the receptors to recover only 0.67 log unit, and a slow component of light-adaptation covering 0.33 log unit might be expected to occur as pigment moved inward. The maximum range over which pigment controls sensitivity during dark-adaptation is about three log units (Bernhard and Ottoson, 1964). Making the most generous allowance which is justified by present evidence, therefore, a slow component of light-adaptation based on a non-linear relation between ΔI and I might be expected to cover as much as one log unit. With the possible exception of two animals, all of the moths studied did not exceed this value. In the two apparent exceptions, light adaptation was not followed by a control dark-adaptation, so that the possibility of physiological deterioration cannot be eliminated.

The experiments reported here lend support to the view that the presence of migratory shielding pigment in the compound eyes of arthropods extends the dynamic range of these eyes and is an adaptation for vision under scotopic conditions.

We are grateful to Mrs. Wiliam Palumbo for her skillful assistance, particularly with the histology; to Dr. Raimon Beard for several gifts of *Galleria* stock; and to Nathan Mandell for aid in the construction of equipment.

SUMMARY

1. An energy flux at the cornea of 5×10^3 ergs sec.$^{-1}$ cm.$^{-2}$ at 500 mμ causes almost complete migration of the accessory shielding pigment in the eye of the wax moth, *Galleria mellonella*. Movement requires approximately 30 minutes. Lower energies bring the pigment to steady-state positions intermediate between the fully light- and dark-adapted positions.

2. Light-adaptation, unlike dark-adaptation, runs a faster time course than pigment migration and is frequently complete in several seconds. This confirms an earlier report of Höglund. The theoretical reasons for a rapid rate of light-adaptation in the face of slow pigment migration are discussed and are shown to be a consequence of (a) equal absorption of the test and adapting lights by the screen of accessory pigment, and (b) a linear relation between ΔI and I.

3. It is suggested that the terms, superposition and apposition eye, be replaced by scotopic and photopic eye, as the latter indicate more accurately the physiological properties of these photoreceptors.

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A DIGENETIC TREMATODE, BOTULUS CABLEI, N. SP., FROM THE STOMACH OF THE LANCETFISH, ALEPISAURUS BOREALIS GILL, TAKEN IN THE SOUTH PACIFIC[1]

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Guiart (1938) described a new genus and species, *Botulus alepidosauri,* on the basis of two specimens from the intestine of *Alepisaurus ferox* taken July 1, 1897, at a depth of 2480 meters in the Atlantic Ocean south of the Island of Madeira. One of the worms, 27 mm. long, 7 mm. wide and 6 mm. thick, was photographed and later lost through desiccation; when found it measured only 18 mm. in length, 5 mm. in width and 4 mm. in thickness. Attempts to restore it were fruitless but the contraction disclosed the uterine loops which occupied "toute la région moyenne du corps." The other specimen was "en assez mauvais état de conservation et avait été détérioré, sans doute lors de l'ouverture du tube digestif de l'hôte." It was sectioned for histological study, but the two photomicrographs showing cross-sections, taken near the middle and near the posterior end of the body, disclose the disintegration of the tissues. Owing to the condition of the material, the description of the species is superficial and incomplete; no measurements are given other than the external dimensions of one specimen; and it is impossible to recognize specific characters. To receive the genus *Botulus,* Guiart erected a new family, Botulidae, noting that it has the external characters of the Azygiidae and the internal characters of the Hirudinellidae. Accordingly, Guiart placed the new family between the Azygiidae and the Hirudinellidae.

Several trematodes were found in the stomachs of *Alepisaurus borealis* taken January 23, 1963, at Station #12 (26° 04.9′ S. and 101° 47.0′ W.) near Easter Island in the South Pacific by the Shoyo Maru Cruise of the Inter-American Tropical Tuna Commission. Eleven specimens were submitted by the collector, Mr. W. L. Klawe, to the writer for study and identification. Subsequently, two additional specimens were received from Mr. Klawe. They were taken November 5, 1963, at station #13 (30° 57.9′ S. and 127° 0.2.5′ W.) by Mr. Eric D. Forsbergh. Grateful acknowledgment is made to Mr. Klawe for his kindness. These worms are obviously members of the genus *Botulus,* and since they cannot be determined as specifically identical with *B. lepidosauri,* they are identified as a new species, *Botulus cablei,* in honor of Professor Raymond M. Cable and in recognition of his contributions to knowledge of the Trematoda.

Two of the specimens were stained and mounted *in toto,* three were sectioned serially, two transversely and the other frontally; while another was dissected by the use of fine needles and pins. All are gravid and in each the vitellaria are visible as a broad longitudinal dark stripe on the ventral side of the body.

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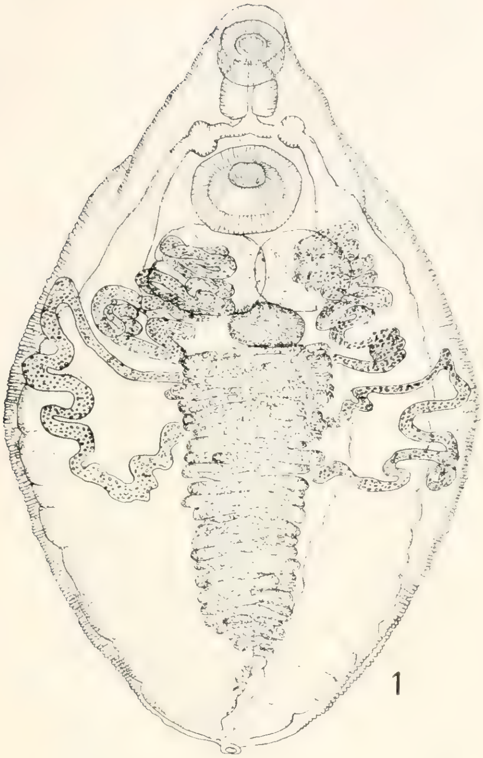
[1] This investigation was supported by NSF G-23561.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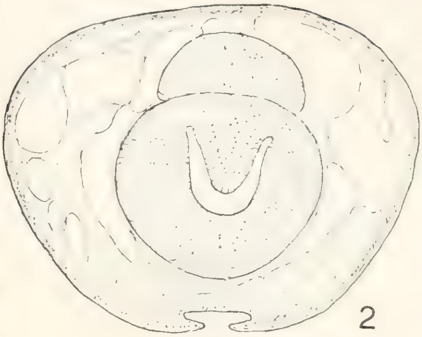
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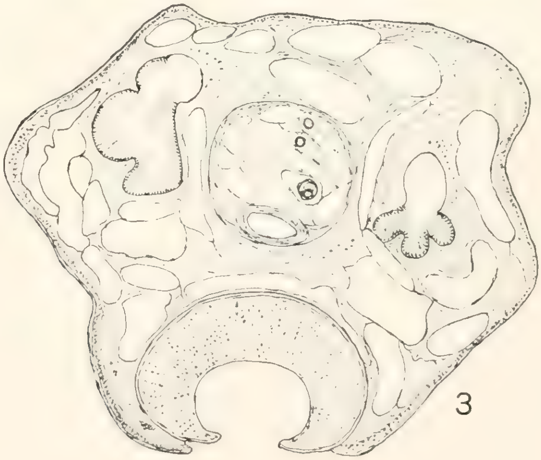
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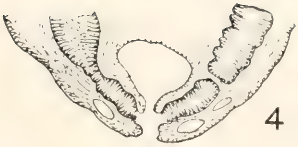
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FIGURE 1. Whole-mount, type specimen, 13 mm. long.

FIGURE 2. Cross-section through the oral sucker at the level of the genital pore. The pharynx appears above the oral sucker.

FIGURE 3. Cross-section through the anterior part of the acetabulum, showing the junctions of the cuticular lined limbs of the esophagus and the epithelial lined digestive ceca, and the genital sac with sections of the metraterm (ventral) and the ejaculatory duct.

FIGURE 4. Frontal section through the junctions of the digestive ceca and the excretory vesicle.

The worms (Fig. 1) are ovate to pyriform to fusiform; either end may be bluntly rounded or attenuated to a pointed tip. The body is only slightly flattened dorsoventrally and is broadly oval (Figs. 2, 3) in cross-section. The largest worm is 33.5 mm. long and 12 mm. in greatest width. It is spindle-shaped, attenuated at both anterior and posterior ends. The next largest specimen is 23 mm. long and 11 mm. wide. The sides are almost parallel; it is rounded posteriorly and tapers sharply at the anterior end. The smallest specimen is 11 mm. long and 6.5 mm. wide, broadly ovate with a pointed anterior tip.

The acetabulum is situated about one-fifth of the body length from the anterior end; it measures 1.44 to 2.25 mm. in diameter and the wall is 0.35 to 0.40 mm. in thickness. The cuticula is heavy, 0.15 to 0.25 mm. in thickness; it is unarmed, although rows of fine, often imbricated, transverse ridges may simulate spination. The surface often presents a granular or pebbled-leather appearance, with refringent dots, but these points are apparently the openings of subcuticular glands. The excretory pore is terminal and the tip of the vesicle may protrude as a papilla. The vesicle extends anteriad, but the exceedingly vacuolated parenchyma makes it impossible to work out the excretory pattern.

The body wall is strongly muscular, especially in the anterior portion of the worm; it is composed of external circular, medial longitudinal, and internal diagonal layers. The longitudinal layer is especially developed, sometimes measuring as much as 0.094 mm. in thickness and composed of six to eight superimposed bundles of fibers. The parenchyma is highly vacuolate, with large fluid-filled cavities.

The mouth is subterminal, the oral sucker is 0.09 to 1.5 mm. in diameter with strong muscular walls (Fig. 2). The lumen is triangular with the ventral apex directed toward the mouth. The sucker is partially overlapped and partially continuous dorsally and posteriorly with the pharynx, which is almost as large as the oral sucker. The lumen of the pharynx is rhomboid to cruciform and the esophagus arises from the dorsal median crus. The esophagus is short and divides into two limbs that diverge laterally, expand to form the two heavy-walled "crops" or "stomachs," and continue laterad and posteriad to open into the digestive ceca (Fig. 3). The esophagus is lined with cuticula and the ceca by a high columnar epithelium whose free edges are filamentous. Posterior to the gonads, the ceca become voluminous, often with folded walls, but they are constricted posteriorly, where their tips communicate with the excretory vesicle (Figs. 1, 4).

The genital pore is located anteriorly and ventrally, a short distance behind the mouth. The [illegible] long genital atrium extends to the level of the posterior end of the pharynx and terminates in a dorsally located protrusible and retractile papilla which bears the openings of the male and female genital ducts. The testes are somewhat ventral, opposite or somewhat oblique with the left one slightly anterior and dorsal to the right, overlapping medially, and situated immediately posterior to the acetabulum. Sperm ducts arise from the dorsal, anterior, medial faces of the testes; they pass forward and mediad and join above the posterior border of the acetabulum, to form the seminal vesicle, an expanded, thin-walled tube, filled with spermatozoa, which coils about above the acetabulum. It loops posteriad and turns forward where it is enclosed by a layer, 0.11 to 0.15 mm. in thickness, of deeply staining secretory cells, and surrounded by a fibrous covering. This prostatic section of the male duct coils anteriad and dorsad, then loses its glandular covering and as a coiled ejaculatory duct, together with the metraterm, enters a muscular-walled sac (Fig. 3), which extends forward and terminates in the papilla which protrudes into the genital atrium.

The ovary is oval, 1.00 to 1.60 mm. long; 1.50 to 1.75 mm. wide; and 1.4 to 1.6 mm. deep, situated slightly left of the median plane. The oviduct arises at the median, posterior face of the ovary and winds ventrad where it receives a duct from the vitelline receptacle and then anteriad and dorsal to expand into the ootype, surrounded by the cells of Mehlis' gland, which measures about 0.50 mm. in lateral and 1.00 mm. in anteroposterior and dorsoventral dimensions. As the duct reaches the dorsal portion of the gland, Laurer's canal diverges and coils dorsally, opening to the surface of the body in the midline above the posterior border of the ovary. The uterus begins at the emergence from Mehlis' gland and the initial coils are filled with masses of spermatozoa. The uterus winds posteriad and laterad, the loops become longer to extend from one side of the body to the other, dorsally and ventrally, occupying the middle third of the body and extending forward on either side of the gonads to the level of the acetabulum. Here the wall of the uterus, which previously was very thin and flexible, becomes thick and muscular, and as the metraterm, passes forward to enter the muscular sac which encloses it and the ejaculatory duct (Fig. 3). The vitellaria are exceedingly numerous and filamentous, extending in a tangled mass on the ventral side of the body, posteriad in the central or median field at least four-fifths of the distance from the ovary to the posterior end of the body. Anteriorly the vitellaria converge and form a vitelline receptacle from which a short and narrow duct opens into the oviduct. The eggs are oval, thick-shelled, operculate and without filaments. They are embryonated when passed and measure 0.030 to 0.034 by 0.023 to 0.025 mm.

The description of *Botulus alepidosauri* by Guiart is so incomplete that it is difficult to recognize the species. The textual account deals with generic characters and the specific features must be derived from the figures. Figure 5 portrays a sagittal section of the anterior region of the body and is highly schematic; Figures 26 and 27 give little precise information. According to Figure 5, the pharynx is much smaller than the oral sucker and quite distinct from it, and the testes are represented as one behind the other. Figure 26 suggests that the vitellaria are dorsal in position, but it is probable that the ventral side of the section is uppermost in the plate. Since the present specimens do not conform to the description of *B. alepidosauri*, they cannot be assigned to that species. The worms are clearly members of the genus *Botulus* and are described as a new species, *Botulus cablei*. Type, cotype, and other specimens are deposited in the U. S. National Museum, Helminthological Collection, No. 60934.

Discussion

Guiart reported the specimens of *B. alepidosauri* were from the intestine of the host, but noted resemblance between them and members of the Azygiidae and the Hirudinellidae. The finding of the worms in the intestine, both dead and one partially disintegrated, indicates that the fish had been dead for some time and that the worms had migrated from the stomach. The members of both the Azygiidae and Hirudinellidae are stomach-parasites of fishes, but they are only distantly related and their similarity is clearly a result of convergence. Accordingly, the allocation of the family Botulidae to a position intermediate between the two families is irrelevant. Actually, the morphology of *Botulus* agrees completely with the characters of the family Hirudinellidae Dollfus, 1932, as given by Yamaguti (1958), and permits the inclusion of *Botulus* in that family. Meanwhile the family Botulidae is reduced to a subfamily, Botulinae.

According to a report in Helminthological Abstracts, 30: 355 (1961), A. S. Skriabin (1958) described a dinurid trematode from *Alepisaurus aesculapius* taken in the Pacific Ocean as a new genus and species, *Profundiella skrjabini.* The specimens differed from members of the Prosorchiinae Yamaguti, 1934, family Hemiuridae, in the position of the testes and the ovary and in the presence of so-called "stomachs" in the digestive system. These features were predicated as the basis for a new subfamily, Profundiellinae. But where these specimens differed from the Prosorchinae, they agree with the genus *Botulus* and there is a possibility that the two generic concepts are identical. In that event *Profundiella* will disappear as a synonym. Morphological agreement appears sufficient to warrant inclusion of *Profundiella* in the subfamily Botulinae, with suppression of the subfamily Profundiellinae.

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ON THE LIFE–HISTORY OF RHIZOSTOME MEDUSAE. III. ON THE EFFECTS OF TEMPERATURE ON THE STROBILATION OF MASTIGIAS PAPUA

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The fact that water temperature has a great deal to do with the initiation of strobilation has been reported for some time. However, most of the cases known are on *Aurelia* and *Chrysaora* etc., in which the onset of strobilation is correlated with low temperature (Berrill, 1949; Thiel, 1962). Contrariwise, it is known that *Cassiopea* and *Cotylorhiza* strobilate in summer (Berrill, 1949). *Mastigias papua* belongs to this group (Uchida, 1926; Sugiura, 1963). At the same time, the present author reported an indispensability of symbiosis of zooxanthellae for strobilation (1964). But in *Mastigias papua,* too, since temperature effect on the metamorphosis is evident, the case will be described in this report.

Materials and Methods

In the present work, two sets of temperature measurements were performed. (1) By following change of the temperature of natural sea water, the temperature at which the ephyrae made the first appearance was determined. (2) In the laboratory, the efficacy of the induction of strobilation under different temperature was examined. As reported in a previous paper (Sugiura, 1964), in the scyphistomae of *Mastigias papua* which have been reared in a zooxanthella-free condition, strobilation can be induced by supplying the symbionts in the culture. Temperature effect on such an induction was studied.

For approximate regulation of temperature, rearing boxes provided with electric bulbs were used. For finer control of temperature, incubators and a constant temperature room were utilized.

Considering the necessity of illumination for maintaining a healthy condition of zooxanthellae, scyphistomae were continuously exposed to the light from a fluorescent lamp during the temperature experiments.

Results

Sea water temperature and strobilation in nature

Every year, the earliest appearance of ephyrae of *Mastigias papua* in the vicinity of the Misaki Laboratory is early summer. According to the survey of 1962, it was July 11. On July 25, abundant ephyrae, which seemed to have been released recently, were obtained (Sugiura, 1963).

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Simultaneously with these observations, temperature measurements were made on the habitat. Notwithstanding the fact that the scyphistomae of *Mastigias papua* have not been found in nature so far, it may not be unjustifiable to consider that the scyphistomae must be living on the bottom of the spot where ephyrae begin to appear, particularly because the area is very limited in the innermost part of the bay. Considering that sea water temperature to be related to strobilation must be that at the bottom of the bay, measurements were taken at 1.5–3.0 m. in depth in the bays of Aburatsubo and Moroiso. The sea water temperature during the first week of the appearance of the ephyrae turned out to be 23° C. (Table I).

However, taking into account the results of indoor experiments to be given in a following section, and also the possibility that the beginning of strobilation in nature could be at least a week before the sighting of the ephyrae, the bottom temperature for strobilation was judged to be about 22° C.

TABLE I

Sea water temperature at the time of the first appearance of the ephyrae

July 11	July 18	July 25	July 31
23.0° C.	23.0° C.	26.5–27.0° C.	26.0–29.0° C.

Effects of rearing temperature for the strobilation in scyphistomae with zooxanthellae

Schyphistomae having zooxanthellae, with previous history of strobilation, were reared from late autumn of the year until the next spring in boxes kept several degrees warmer than room temperature by electric bulbs. Three grades of temperature were tested. The procedure was as follows: Test organisms were put in three boxes of the same size and although temperatures in the boxes were allowed to fluctuate with the room temperature, box A was kept about 10° C. higher constantly and B a little less higher and C still closer to the room temperature. In reality the lowest temperature in A was 19° C., in B it was 17° C. and in C about 15° C. when the room temperature was around 10° C.

When the water temperature rose with the season, initiation of strobilation first occurred in A (March 21), and a month later (April 15–24) in B in 6 dishes except one B_7 group, while no strobilation had taken place in C by that time. Water temperature at the beginning of strobilation was 20–22° C. in both A and B.

Next is a question how the result will be, if the culturing temperature is never allowed to exceed 20° C. For this purpose, the culture C was transferred to the constant temperature room of 20° C. at the middle of April before the atmosphere reached that temperature level. This group had not strobilated at least until June 25 when B_7 did. At this point (June 25), C was further divided into two classes, one being left at 20° C. while the other was returned to room temperature. The former did not strobilate all through the summer. In the latter group, however, the first strobilation took place on August 7.

In conclusion, 20° C. seems to be just below a critical level and culturing temperature has to go beyond this threshold in order to stimulate strobilation. On the basis of the almost perfect agreement between the data obtained in nature and in the laboratory, it is possible to say that 22° C. is the lowest effective temperature for inducing strobilation. Needless to say, in zooxanthella-free scyphistomae, even much higher temperatures are quite ineffective for causing strobilation.

Temperature higher than 22° C.

For studying the effect of temperature higher than 22° C., it was unavoidable to collect individuals which have failed, for some reason or another, to react to 22° C. with strobilation. Using these organisms, a part of them (group a) was maintained at 28–29° C. for 20 days and the remainder (group b) was first set back to 20° C. for a month (Sept. 21–Oct. 21) and then to 28–29° C. In group a, no strobilation occurred in all five dishes, while among group b, strobilation began within 10 days after raising the temperature in 4 dishes out of 5.

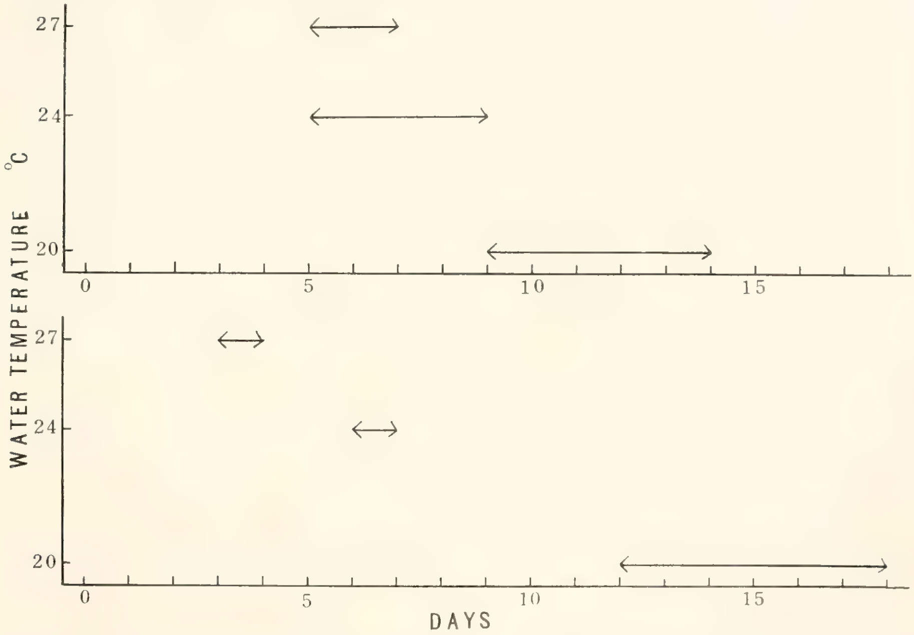

FIGURE 1. The effect of water temperature on two steps of strobilation. Upper chart: Latency between symbiosis and the beginning of morphological change for strobilation. Lower chart: Latency between the beginning of metamorphosis and the release of the ephyrae.

In order to confirm the above situation further, the group a and the group b were mixed and scyphistomae were randomly divided into two parts and the two parts were equally subjected to 20° C. for a month at first (Nov. 26–Dec. 30) when one part was transferred to 25° C. and the other part kept at 20° C. In the part made warmer, strobilation took place in all 4 dishes within a month, while in the cooler part, strobilation was seen in none of 4 dishes.

Judging from the above experiments, when scyphistomae fail once to react to 22° C., a further increase of the culturing temperature has no effect for causing stimulation. Instead, the temperature should be lowered once more for nearly a month before raising it, during which time the organisms seem to be made receptive to a warm temperature. At any rate, the main point of this finding is that it is now possible to induce strobilation by regulating the temperature, regardless of the season.

Temperature effects on separate steps of the process of strobilation

When scyphistomae which have over-wintered in a zooxanthella-free condition are given the symbionts, they strobilate immediately (within several days) with a high degree of reliability. Moreover, they can sometimes strobilate at 20° C., showing their sensitivity has been heightened by a long sojourn in a low temperature. Using such materials, the effects of 27°, 24° and 20° C. were examined concerning the latency between supplying the symbionts and the beginning of strobilation and that between the onset of strobilation and the release of ephyrae (Fig. 1). As is clear from the figure, the latter step is more sensitive to change of temperature. Since the former step mostly corresponds to the period of multiplication of symbionts, this may mean that zooxanthellae are less sensitive to temperature change than the medusa itself.

The writer wishes to express his thanks to Prof. K. Dan for his constant guidance in the course of the work.

SUMMARY

1. In the habitat, the first appearance of the ephyrae coincides with the sea water temperature of about 22° C.

2. In the laboratory, the minimum effective range of temperature for induction of strobilation by giving zooxanthellae is found to be 20–22° C.

3. Individuals with zooxanthellae, which have once failed to react by strobilation by 20–22° C., can no longer be induced for strobilation by a further rise of temperature.

4. These individuals do react if they are cooled to 20° C. for about a month and then exposed to the higher temperature.

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PHYSIOLOGY OF INSECT DIAPAUSE. XV. THE TRANSMISSION OF PHOTOPERIOD SIGNALS TO THE BRAIN OF THE OAK SILKWORM, ANTHERAEA PERNYI[1]

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The pupa of the oak silkworm, *Antheraea pernyi,* is enveloped in a stout-walled cocoon which gives every impression of being opaque. It seems incredible that an insect in this situation could be influenced by the length of the day and night. Yet such is indeed the case. As demonstrated in the previous paper of this series (Williams and Adkisson, 1964), diapause is persistent at 25° C. when cocoons are exposed to short-day conditions (daily photophases of 4 to 12 hours). By contrast, photophases of 15 to 18 hours provoke the termination of dormancy and the initiation of adult development. It was possible to show that the sensitivity to photoperiod depends on the direct action of light on the pupal brain. In some unexplained manner, light of appropriate wave-length is able to penetrate the opaque cocoon and the pupal cuticle to act on the brain itself.

The phenomenon is examined in detail in the present investigation. All experiments were performed on *A. pernyi.* The cocoons were the diapausing first-brood harvested in late July; on August 27 they were shipped from Japan in a series of cardboard boxes which arrived at Harvard University on September 30. The cocoons were spread on tables and stored at 25 ± 0.5° C. in a room programmed for a daily illumination of 8 hours.

EXPERIMENTAL RESULTS

1. *Photoperiod responses of naked pupae versus pupae in cocoons*

Ninety-six diapausing pupae were removed from cocoons. Forty-eight were placed in an incubator programmed for a daily 8-hour photophase at 25° C.; the other 48 were placed in a similar incubator programmed for a 16-hour photophase. Each incubator also received 92 unopened cocoons from the same lot of material. The cocoons were spread on the shelves and thereby exposed to fluorescent illumination having an average intensity of 175 foot-candles (1883 lux).

The naked pupae were inspected once a week to detect the initiation of adult development in terms of the retraction of the wing epithelium. In the case of the intact cocoons, the emergence of the moths was noted daily; the initiation of adult development was computed as having occurred 20 days prior to adult emergence (Williams and Adkisson, 1964).

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The results are summarized in Figure 1. During the 8-week term of the experiment, diapause was persistent in all naked pupae and intact cocoons exposed to the short-day regimen (horizontal line in Figure 1). By contrast, the entire series of animals exposed to the long-day conditions initiated development. As indicated by the upper pair of curves in Figure 1, pupae in cocoons responded just as promptly to the long-day stimulus as pupae removed from cocoons. This finding shows that, despite the relatively low intensity of the incident illumination, the presence of the cocoon did not curtail the sensitivity to photoperiod.

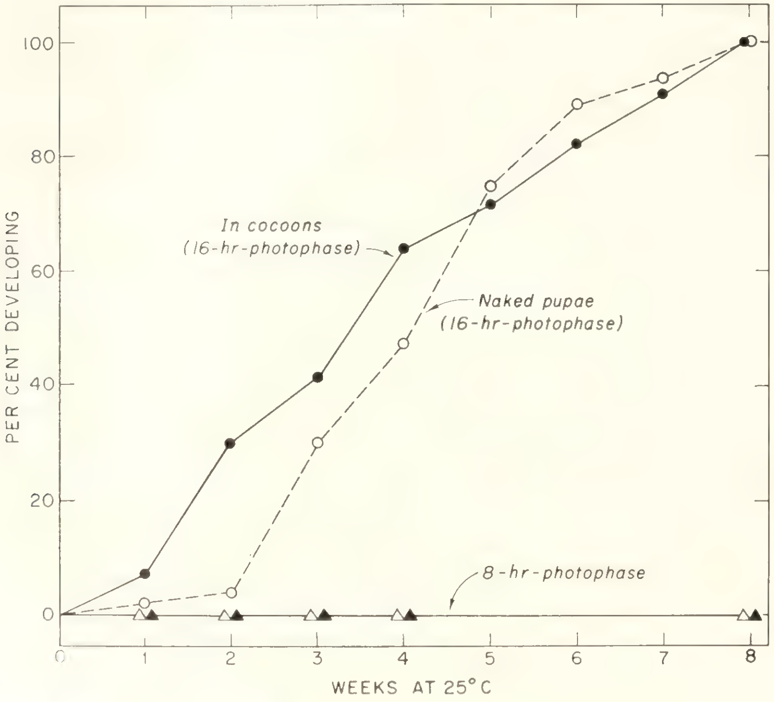


FIGURE 1. The effects of long- and short-day photoperiods on the termination of pupal diapause by naked pupae and by pupae in cocoons. All individuals exposed to the long-day regimen initiated development (*top two curves*). None initiated development when exposed to the short-day regimen (*bottom line*).

2. *Spectral sensitivity of diapausing Pernyi pupae*

Fifty diapausing pupae were removed from cocoons and ten were placed head-up in each of five depressions of an aluminum "muffin tin." Arrangements were made for positioning Corning filters over the depressions. For this purpose each 8 × 8 cm. filter was sealed with masking tape to a 2-cm. collar cut from a 7.5-cm. thin-wall brass pipe. Each filter was positioned over a specific group of pupae and sealed in place with a gasket of plasticene.

With the filters removed, the entire assembly was placed at 25° C. in an incubator programmed for a daily 16-hour photophase. After 8 hours of exposure to the unfiltered light, the filters were sealed in place so that the pupae received filtered-light during the final 8 hours of the 16-hour photophase. The filters were removed the following morning at the outset of the next photophase. This regimen was repeated daily for 8 weeks.

The five filters, together with their peak transmissions, were as follows: Corning color specification No. 5–62 (398 mμ), No. 5–74 (434 mμ), No. 4–105 (508 mμ), No. 3–110 (580 mμ), and No. 2–78 (640 mμ).

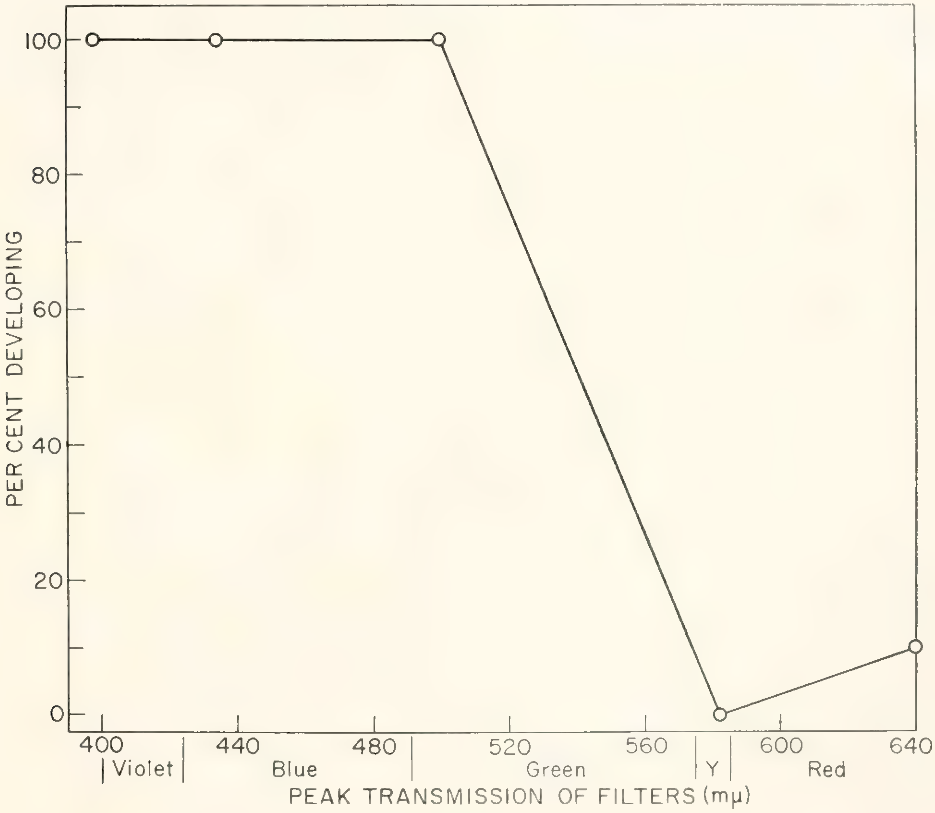


FIGURE 2. The spectral sensitivity of diapausing pupae of *A. pernyi*. The wave-lengths effective in causing the termination of diapause include violet, blue, and blue-green light.

Each of the five groups of pupae was inspected daily in order to detect the initiation of adult development. As controls, two groups of ten pupae were exposed to white light at 25° C. in incubators programmed for 8- and 16-hour photophases, respectively. During the 8-week term of the experiment, these controls behaved as follows: all the animals exposed to white light for 16 hours per day initiated development, while none initiated development under the short-day regimen.

The results observed in the five experimental groups are summarized in Figure 2. Diapause was terminated by all pupae exposed to violet, blue, or blue-green light (398 mμ to 508 mμ); indeed, these animals initiated development as promptly as the control series that received 16 hours of unfiltered light. By contrast, diapause was persistent in all pupae exposed to yellow light (580 mμ) and in all but one of the pupae exposed to red light (640 mμ).

3. *β-Carotene-filtered light*

Because of the involvement of carotenoids in so many photic reactions, a series of filters was prepared containing graded concentrations of β-carotene dissolved in 95% ethanol. The solution was placed in optical flats prepared by clamping a 0.5-cm. Lucite ring between pairs of glass plates. The filters contained β-carotene in final concentrations of 1000, 100, and 4 parts per million; the densest filter was a deep amber-yellow; the least dense was almost colorless.

The experiment described in the preceding section was repeated using these filters. All individuals initiated adult development at approximately the same rate as controls exposed to a 16-hour photophase of unfiltered light. This shows that β-carotene does not remove all wave-lengths effective in the photoperiod response.

At the conclusion of the experiment, the absorption spectrum of each carotene solution was measured. Even the most concentrated solution showed substantial transmission at wave-lengths higher than 470 mμ. Therefore, the carotene-filtered light contained wave-lengths (470–508 mμ) which were fully effective in implementing the photoperiod response of naked pupae.

4. *Spectrophotometric measurements*

A series of nine Kodak neutral density filters (0.9 ND) was calibrated for light transmission at wave-lengths ranging from 400 mμ to 700 mμ. A Zeiss M 4 Q III spectrophotometer was utilized for this purpose, the filter being placed in the experimental beam and its transmission measured against air. Transmission by the filters proved to be a function of wave-length and varied systematically from [illegible] to 11%.

A stack of five ND filters was placed in the experimental beam and its transmission measured against a stack of four filters placed in the reference beam. The difference in light transmission at all wave-lengths corresponded to that of a single filter. This demonstrated that the Zeiss instrument was sufficiently sensitive to measure transmissions by objects as dense as five 0.9 ND filters.

A flattened fragment of a cocoon was positioned in the experimental beam and its transmission compared at each wave-length with that of a stack of four ND filters placed in the reference beam. The transmission of the cocoon was then calculated for each wave-length.

The results summarized in the lower curve in Figure 3 confirm that the cocoon is an extremely dense object to the simple transmission of light; thus, at 400 mμ the transmission was [illegible]; at 700 mμ, 0.014%. By this same procedure, the transmission was measured for the following fragments of pupal cuticle: (1) the unpigmented facial cuticle; (2) the tan wing cuticle of a palely pigmented pupa; and (3) the black wing cuticle of a heavily pigmented pupa. In these measurements one or two ND filters were placed in the reference beam. The results recorded as the top three curves in Figure 3 show the pupal cuticle to be 5000-fold more transparent than the cocoon to the effective wave-lengths. Moreover, the transmission of blue light (460 mμ) by the facial cuticle was 2 to 5 times that of the wing cuticles.

This fact is illustrated in Figure 4. The anterior third of a Pernyi pupa has here been eviscerated and illuminated from behind to show the transmission of light through the unpigmented facial cuticle and, to a lesser degree, through the tan cuticle of thorax, legs, and antennae.

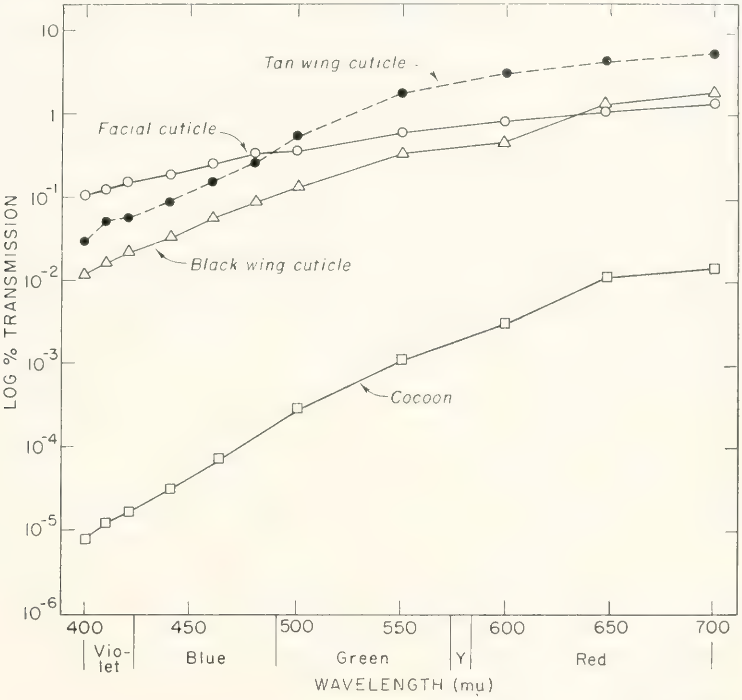


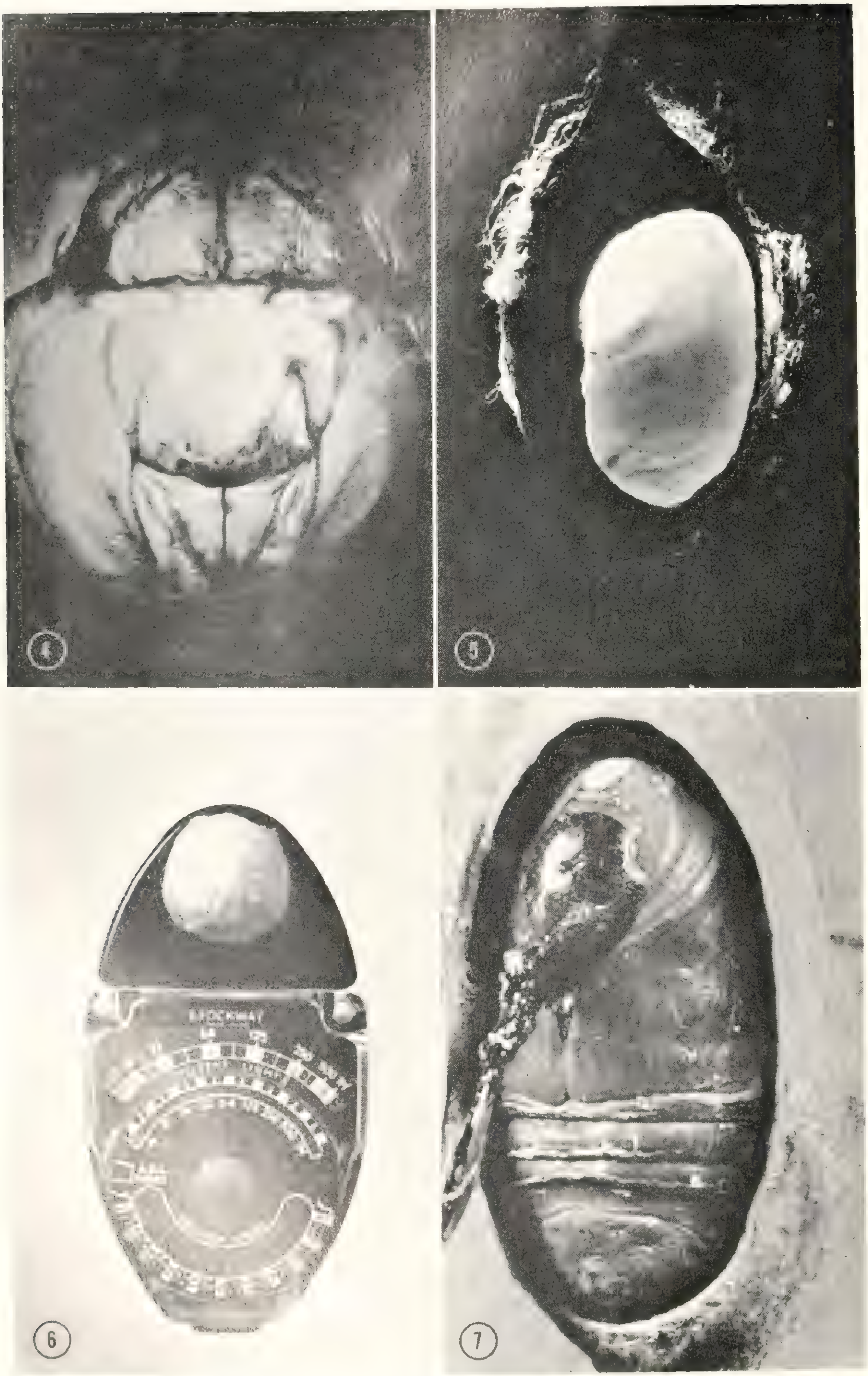
FIGURE 3. Spectrophotometric studies of light transmission by the cocoon (*lower curve*) and by three types of pupal cuticle (*top curves*).

By a combination of the spectrophotometric measurements on the cocoon and the facial cuticle, we calculate that, of blue light (460 mμ) incident on the outside of the cocoon, only 0.0000003% could reach the brain by simple transmission.

5. *The cocoon as a light-integrating sphere*

Since the cocoon is essentially an opaque object in terms of the transmission of unscattered light, the demonstrated light-sensitivity of the pupa must depend on the collection and integration of scattered light (Jacquez and Kuppenheim, 1955).

The geometry of the cocoon proves to be little short of optimal for this purpose. This fact is illustrated in Figure 5 where an empty cocoon has been illuminated



Figures 4–7.

from behind by the focussed beam of a 100-watt zirconium arc lamp. By excising the proximal face of the cocoon, one can witness the generalized illumination of the cavity of the cocoon.

This same phenomenon is also illustrated in Figure 6. Here, the light-integrating hemisphere of an incident-light exposure meter has been removed and replaced by the upper half of a Pernyi cocoon. A significant deflection of the meter is observed when the cocoon is illuminated. In order to describe this phenomenon in quantitative terms, the following experiment was performed:

A cocoon was opened by a circumferential razor-blade cut around its long axis. After the removal of the pupa and the old larval skin, a micro-photocell (Texas Instruments LS-222) was suspended in the cavity of the cocoon. The latter was reassembled and sealed with Duco cement and a narrow band of opaque tape; the paired leads to the photocell passed to the outside through the incision.

The cocoon was suspended in a dark room at a standard distance from a microscope lamp (Bausch and Lomb No. SVB-73). The light first traversed a water-filled filter, 3 cm. in depth, and then illuminated one entire 180° hemisphere of the cocoon. The output of the photocell was measured in millivolts. The measurements were repeated with the cocoon oriented at various attitudes with reference to the incident white light. The output of the photocell varied from 120 to 270 millivolts, depending on the orientation of the cocoon, the average being about 240.

The photocell was then removed from the cocoon and illuminated directly by the same white light at the same standard distance. The output was now 400 millivolts. Neutral density filters were then placed in the light beam to lower its intensity. A sandwich of two filters, each having 10% transmission for white light, decreased the photocell output to 240 millivolts. So, in rough terms, we can say that about 1% of the incident white light reached the photocell when the latter was inside the cocoon.

6. *Light-integration as a function of wave-length*

The preceding experiment was repeated with interference filters placed in the beam to establish monochromatic light. A total of nine wave-lengths was examined in this manner. Then with the photocell removed from the cocoon, the latter's properties at each wave-length were equated to that of the calibrated ND filters.

The results are recorded as the upper curve in Figure 8. The light-integrating properties of the cocoon prove to be substantial, especially in the range 440 mμ to 510 mμ.

FIGURE 4. The anterior third of a Pernyi pupa has been eviscerated and illuminated from behind by white light. The photograph illustrates the transmission of light by the unpigmented facial cuticle and, to a lesser degree, by the tan cuticle of the thorax, legs, and antennae.

FIGURE 5. An empty cocoon is here illuminated from behind by an intense, focussed spot of white light from a 100-watt zirconium arc lamp. By excising the proximal face of the cocoon, one can witness the collection of scattered light within the cavity of the cocoon.

FIGURE 6. A hemisphere of Pernyi cocoon serves as a light-integrating sphere when substituted for the integrating hemisphere of an incident light exposure meter.

FIGURE 7. A micro-photocell has been sealed into a Pernyi pupa with its light-sensitive element placed just beneath the unpigmented facial cuticle. The pupa was then sealed inside the cocoon to permit measurements of the light energy reaching the brain at each wave-length.

7. *Light reaching the brain*

The cuticle overlying the legs was excised from an anesthetized pupa. The micro-photocell was then implanted through this mid-ventral area so that its light-sensitive element was positioned precisely beneath the transparent facial cuticle, *i.e.*, in the place normally occupied by the brain. The photocell was held

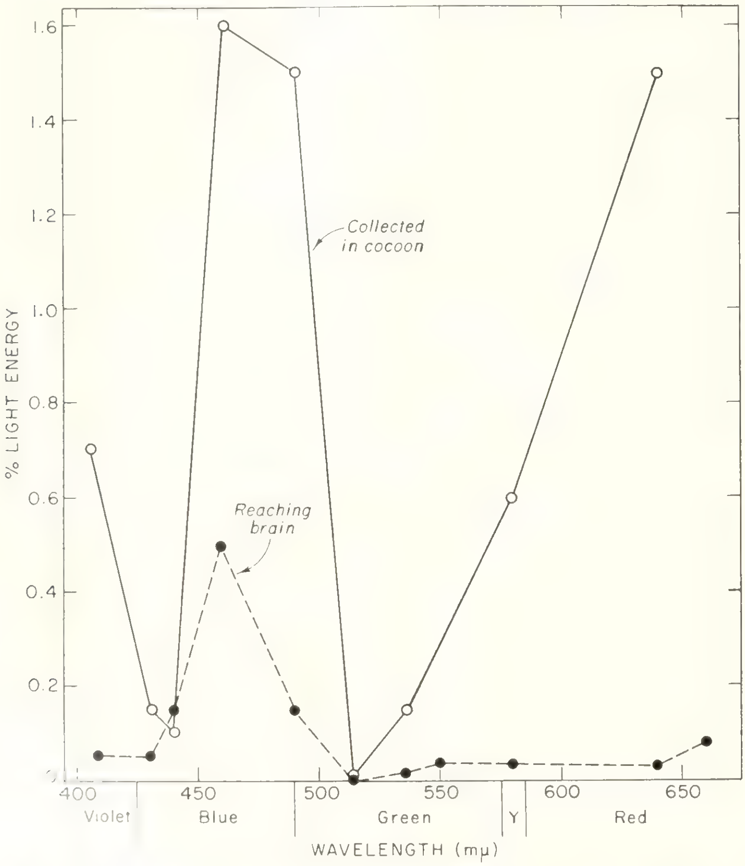


FIGURE 8. [illegible] of wave length on the per cent of incident light energy which penetrated the [illegible] and which reached the brain of a pupa sealed within a cocoon [illegible] system shows a conspicuous "window" centering around 460 mμ.

in place and the wound sealed by the application of opaque cement (see Fig. 7). The pupa was then sealed within a cocoon as described above.

The measurements described under Section 6 were repeated with the results summarized by the lower curve in Figure 8. The optical properties of the entire system prove to be maximally effective for blue light. Thus, in the range 440 to 490, more than 0.14% of the light energy reached the brain; indeed, at 460 mμ, no less than 0.5% of the scattered light was recollected to act on the brain.

DISCUSSION

1. *Spectral sensitivity of the pupal brain*

In the previous paper of the series (Williams and Adkisson, 1964), light was found to act directly on the pupal brain to control the secretion of brain hormone. According to the present study, the effective wave-lengths extend throughout the lower third of the visible spectrum to include the violet, blue, and blue-green (398 mμ to somewhat above 508 mμ). Yellow or red light (580 mμ to 640 mμ) was without significant effects. The lowest wave-length tested (398 mμ) was fully effective; consequently, we are unable to state how far the spectral sensitivity extends into the ultraviolet.

In order to have any effect, the pertinent wave-lengths must be absorbed. And since the effective wave-lengths include the lower third of the visible spectrum, the absorption of light is evidently accomplished by a pink brain pigment.

The spectral sensitivity of the Pernyi brain is in close agreement with that described for the mite, *Metatetranychus ulmi* (Lees, 1955), and for certain, but by no means all, insects that have been studied (for reviews, see Lees, 1955; Bünning, 1960, 1964; Farner, 1961; de Wilde, 1962; Danilevskii, 1965).

The Pernyi pigment therefore differs from the phytochrome system of the higher plants in terms of the latter's sensitivity to red and far-red (Borthwick, 1959; Hendricks, 1959). However, the photoperiodic responses of fungi (Bünning, 1964) and the phototropic reactions of higher plants (Shropshire and Withrow, 1958; Withrow, 1959) show a spectral sensitivity which is essentially the same as that of the Pernyi brain.

The phototropic reactions of plants are thought to depend on the absorption of blue light by a carotenoid or flavin pigment. In the present investigation, β-carotene was tested and found to be inappropriate in the insect system because of its scant absorption of certain wave-lengths (470–508 mμ) which were fully effective in the photoperiod response. However, it may be noted that numerous other carotenoids, such as the arthropod pigment, astaxanthin, show absorption spectra which include these higher wave-lengths (Karrer and Jucker, 1950).

2. *Optical properties of the cocoon and the pupal cuticle*

In order to be absorbed by the pink brain pigment, light must first traverse a series of barriers before reaching the brain; namely, the wall of the cocoon, the pupal cuticle, the underlying epidermis, and a narrow zone of hemolymph. Due to its opacity the cocoon is the major obstacle to the simple transmission of light (Fig. 3). The pupal cuticle is, by contrast, 5000 times as transparent as the cocoon. Moreover, the measurements performed on the several categories of pupal cuticle show that, at 460 mμ, the unpigmented facial cuticle is twice as transparent as tan wing cuticle and five times as transparent as black wing cuticle (Fig. 3).

3. *Light-integration by the cocoon*

Despite its opacity, the cocoon proves to be an effective vehicle for the collection and integration of scattered light ("haze"). The blue haze collected within the cavity of the cocoon then penetrates the pupal cuticle to act on the brain. Analogous biological systems have been discussed in detail by Shibata (1958) and French (1959).

Our measurements reveal the surprising fact that the entire system is remarkably effective in the collection, integration, and transmission of blue light ranging from 440 mμ to 490 mμ. As noted in Figure 8, a particularly prominent "window" centers around 460 mμ. All these wave-lengths, as mentioned above, are fully effective in the photoperiod reaction.

4. *Light intensity*

In the experiments reported here, no attempt was made to obtain a true action spectrum in terms of the energy threshold of the brain as a function of wave-length. The fluorescent lamps in our incubators saturated the brain's photoreceptive mechanism even though the incident illumination on the outside of the cocoons was only 175 foot-candles (1883 lux)—*i.e.*, about 2–5% the intensity of direct sunlight.

Spectroscopic examination of the fluorescent lamps revealed three intense emission lines (435.8 mμ, 546.1 mμ, and 577.0 mμ) of which only the one at 435.8 mμ falls within the spectrum shown to be effective in the photoperiod reaction. If we assume that, of the total light acting on the outside of the cocoon (175 foot-candles), about 25% is contributed by effective wave-lengths, the intensity factor is reduced to 44 foot-candles. Of this incident intensity, not more than about 0.2% could reach the brain after traversing the several barriers (Fig. 8). Hence we may say that the brain's photoreceptive mechanism is fully saturated by intensities not in excess of about 1 foot-candle (10.8 lux) of blue light. This value is in line with those reported for other insects where saturating intensities ranging from 0.01 to 10 foot-candles (0.11–108 lux) have generally been encountered (see Lees, 1955; Farner, 1961; de Wilde, 1962).

SUMMARY

1. The pupal diapause of *Antheraea pernyi* is sustained by exposure of cocoons to short-day conditions (daily photophases of 4 to 12 hours) and terminated after exposure to daily photophases of 15 to 18 hours.

2. The photoperiod signal is conveyed by the direct action of violet, blue, and blue-green light (398–508 mμ) on the brain itself. This finding implicates a pink brain pigment in the absorption of the effective wave-lengths.

3. Despite its opacity, the cocoon functions as a light-integrating sphere in the collection of scattered light—especially of blue light ranging from 440 mμ to 510 mμ.

4. After its collection within the cavity of the cocoon, the blue "haze" penetrates the pupal cuticle to act on the brain. The brain's photoperiod mechanism is "saturated" by less than 1 foot-candle of blue light.

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CTENIDIAL NUMBER IN RELATION TO SIZE IN CERTAIN CHITONS, WITH A DISCUSSION OF ITS PHYLETIC SIGNIFICANCE[1]

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Before the discovery of the living monoplacophoran genus, *Neopilina*, in 1952, any summary diagnosis of the phylum Mollusca included such a phrase as "coelomate, totally lacking metameric segmentation." This was completely acceptable despite the well-known replication of several sets of organs—including shell valves, pedal muscles and ctenidia—in the polyplacophoran Amphineura—the chitons. However, the detailed morphology of *Neopilina galatheae,* so carefully described by Lemche and Wingstrand (1959b, see also Lemche, 1957), led these authors to propose that several features in both *Neopilina* and chitons were primitive metameric characters providing undeniable evidence of the segmental origin of the phylum Mollusca. Certain workers with extensive knowledge of the functional morphology of molluscs have not been able to accept these characters as evidence of metameric origins (Yonge, 1957; Morton and Yonge, 1964), regarding the multiplicity of certain organ systems (of cephalopods and chitons as well as of monoplacophorans) as being replication with functional, rather than segmentation with phyletic, significance. Other authors with extensive molluscan background (Fretter and Graham, 1962) have partially accepted the features as indications of metamerism and to some extent revived an older theory of molluscan origins. This older theory was developed as a result of studies of the genital and excretory systems of chitons and cephalopods (see Pelseneer, 1899, 1906; Naef, 1926) and proposed a stem group of molluscs with short segmented bodies. For many years it seemed that this older theory had been completely dismissed as a result of the work on the functional morphology of primitive gastropods which culminated in an important survey by Yonge (1947), which established as the most highly integrated mollusc a totally unsegmented animal with a posterior mantle-cavity and one pair of [illegible] ctenidia). The description of *Neopilina* has reopened discussion of possible metamerism in primitive molluscs, and, as outlined above, this has again involved consideration of the multiplied organ systems in chitons.

This paper presents data on the length-weight relationship and the ctenidial numbers found in *Chaetopleura apiculata* from Cape Cod and in *Lepidochitona cinereus* from the North Sea coast of Scotland, with some brief notes on conditions in certain other species of chitons. The data form part of other work by the present authors on the functional morphology of post-larval chitons (S. C. B.) and on variations in growth and fecundity in chitons (W. R. H.), but are presented here because of their significance in relation to the alleged metamerism of primitive molluscs.

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MATERIALS AND METHODS

Two species of chitons were studied in detail. *Chaetopleura apiculata* is the commonest chiton of the Atlantic seaboard of the northeastern United States, and was collected from the Northwest Gutter of Hadley Harbor and from Penzance, Buzzard's Bay, both near Woods Hole, Massachusetts. *Lepidochitona cinereus* is one of the four commoner intertidal chitons of British waters, and was collected from rock pools at Leckmoram Ness on the Firth of Forth, near North Berwick, Scotland. Both chitons were collected from between low-water of neap tides and low-water of springs. The species and collection localities of the other chitons not studied in detail are given in the text where they are mentioned. Initial ctenidial counts were made on living chitons and on material fixed and preserved by various standard procedures. The principal material of both *Chaetopleura* and *Lepidochitona* used to establish the length-weight relationship and to count gills was prepared by a method which produces maximum flexibility of the ctenidia. A similar method has been employed extensively by one of the present authors (W. R. H.) in work on slugs and other pulmonate gastropods, and an account of this use was given by Owen and Steedman (1958; see also Rosewater, 1963, for further references). In the case of the chitons, they were placed in fingerbowls of clean sea water and propylene phenoxetol[4] gently added to form globules on the bottom of each dish (*not* touching the chitons). The amount of propylene phenoxetol added was about 0.1–0.2% of the volume of sea water. They were then left undisturbed, in dim light, for about eight hours. Fixation of these partially-narcotized chitons was then carried out in 12% formalin in sea water, each being held flattened against a glass surface when first immersed in the fixative. They required to be held for only about 10–15 seconds to prevent curling. Fixation was continued for four hours, and the chitons then washed for four hours in running water. Finally they were transferred to the storage fluid: an aqueous solution of 1% propylene phenoxetol, and 1% ethyl alcohol in 10% glycerol. Animals prepared in this way have life-like tissues, soft, flexible and excellent for dissection. For the benefit of anyone applying this method to other material, it should be emphasized that propylene phenoxetol has been used only (1) as a relaxing agent, and (2) as a preserving fluid (actually as a bacteriostatic agent). It should be used in the latter capacity only after adequate fixation (since it does not prevent tissue autolysis, etc.). In this work, chiton length was taken as the maximum distance along the midline from the anterior to the posterior "girdle" edges of flattened specimens. Length measurements were made to the nearest 0.1 mm. with an engineer's dial caliper. Weights were "wet tissue weights" determined to the nearest milligram after removing excess external water with filter paper, on a precision micro-torsion balance (V.D.F., Holland) with a scale of 1 gm. in milligrams. Ctenidia were counted under a dissecting microscope at about 20 ×, using high-intensity incident illumination, by leafing over with a fine dissecting needle. All counts were checked, "asymmetrical" chitons re-checked and significant counts double-checked by both

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[4] U. S. distributor: Goldschmidt Chemical Corp., 153 Waverly Place, New York 14, New York.

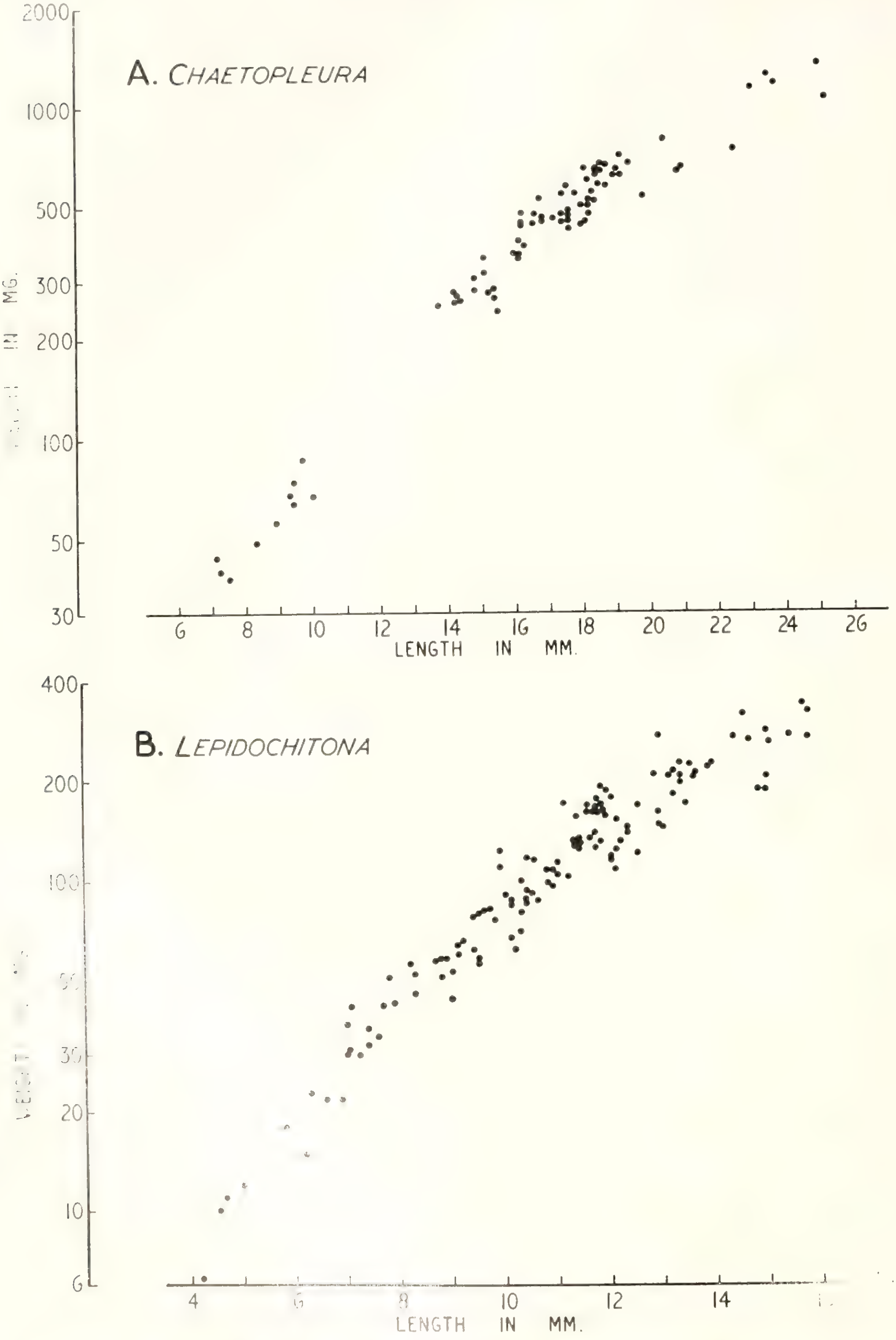

Figure 1. "Wet tissue weight" in relation to body length in chitons (weights in milligrams on the ordinate are plotted logarithmically). 1A, for *Chaetopleura apiculata* from Woods Hole, Mass.; 1B, for *Lepidochitona cinereus* from the Firth of Forth, Scotland.

authors. Those chitons with obvious pallial or pedal damage (or signs of regeneration), and those few with an obvious gap in the gill series, were rejected and not included in the ctenidial counts. "New" ctenidia in the process of formation presented difficulties. Tiny dome-shaped buds lacking any obvious structure *were not* counted, but even the smallest gill rudiments showing differentiation of an axis with two or more ctenidial leaflets *were* counted.

RESULTS

The "best" measures of size and growth in chitons utilize volume or mass. Linear measurements of the whole animals involve several sources of error, including variations in flattening and in the degree of expansion of the mantle edge. Attempts have been made to avoid these errors by using measurement of individual plates (see Arey and Crozier, 1919), or along the plate series. The method of preparation outlined above results in flat, little-contracted chitons whose lengths can be readily and reproducibly determined. Figures 1A and 1B show the relation of length to wet tissue weight for *Chaetopleura apiculata* and for *Lepidochitona cinerea*. The relation of weight to length calculated for *Chaetopleura* is $W = 0.0881\ L^3$, and for *Lepidochitona* is $W = 0.0833\ L^3$ (with W in mg., and L in mm.). That is to say, the shapes and relative densities of *Chaetopleura* and of *Lepidochitona* (and indeed of most other chitons) are closely similar to each other.

These weights are used in Figures 2A and 2B, in which they are plotted against the ctenidial numbers actually counted in each specimen. Bolder points indicate chitons which are symmetrical, *i.e.*, have the same number of gills on the left as on the right side. Horizontal lines indicate asymmetric chitons and link the appropriate gill numbers. In both species there is clearly a correlation between gill number and size. In the sample of *Chaetopleura apiculata* (Fig. 2A) weighed and counted, 58 out of 76 are symmetrical. In some of these numerically symmetrical specimens, however, there is marked asymmetry in the size of the anteriormost gill or gills. In adult specimens of *Chaetopleura,* as in all chitons, these are the most recently formed. Although there is clearly a correlation of increase in ctenidial number with increase in weight (Fig. 2A), there is no direct evidence available to link weight (or any other size measurement) with age in *Chaetopleura apiculata.* Circumstantial evidence suggests that in *Lepidochitona cinereus,* and in other relatively small chitons like *Chaetopleura,* the length of life is between three and six years (see, however, Comfort, 1957). From the sample of *Chaetopleura apiculata* used in Figure 1A and 2A, and from other samples measured during studies on fecundity, the only clearly marked "generation" is made up of specimens less than 12 mm. long, *i.e.*, under 100 mg. weight, in late summer samples. Later generations (200 mg. to 1.5 g. wet weight) are inseparable on presently available data. It should be noted that this group of small chitons (almost certainly about 12 months old in late summer) already have from 17 to 20 gills on either side. There is a gap in our knowledge of *Chaetopleura* between the immediate post-larval stages and these young "year-old" adults. Recent work (S. C. B., unpublished) has shown that no ctenidia are present in the pallial grooves of the 30-day post-larva (which has the full eight shell plates). However, from the sixth day to the 33rd day post-larva, water currents created by ciliated epithelia in the pallial grooves are functionally analogous to those found in the adult, in which they are produced by

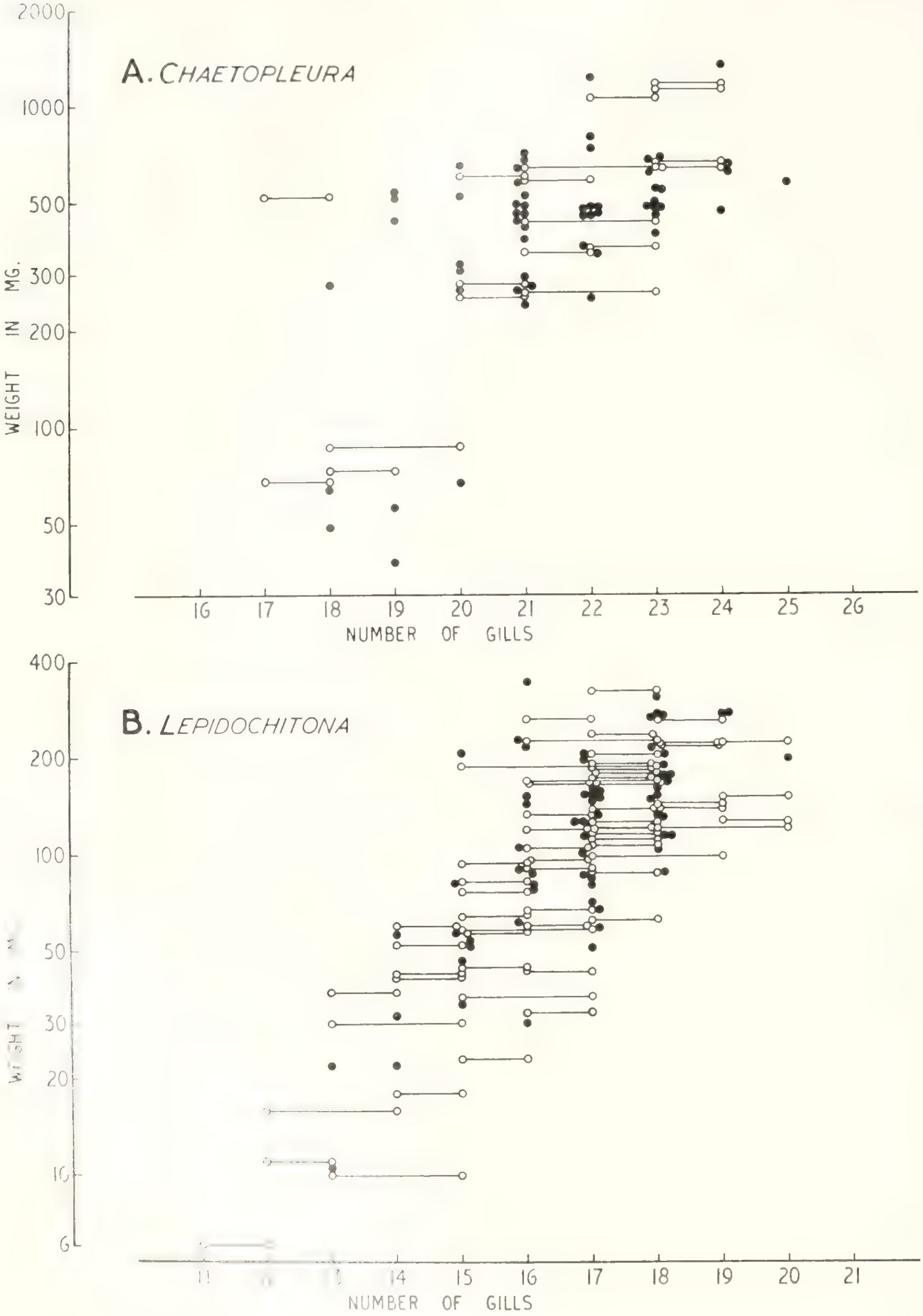
A. CHAETOPLEURA
WEIGHT IN MG.
2000
1000
500
300
200
100
50
30
16
17
18
19
20
21
22
23
24
25
26
NUMBER OF GILLS
B. LEPIDOCHITONA
400
200
100
50
30
20
10
6
14
15
16
17
18
19
20
21
NUMBER OF GILLS

Figure 2.

the lateral cilia on the faces of the ctenidial leaflets. It is hoped to investigate the later post-larval development, particularly with regard to ctenidial morphogenesis and ciliation. Meanwhile, in functional terms, it can be assumed that the surface-volume ratio in the 30-day chiton (measuring 0.55 mm. long, and assessed at 14.4 μg. live weight) is such that the animal has no respiratory "need" to develop the elaborate increase in surface area provided by ctenidia. The last large ctenidium in young adult specimens of *Chaetopleura* is the third from the posterior end in each ctenidial row (*i.e.,* immediately behind the renal opening, and the second gill behind the genital opening). This particular gill is almost certainly the first formed and a definite number (two) of ctenidia are added behind, while a less definite number (from 14 to 22 in our samples) are added anteriorly. Asymmetry in gill numbers involves only the anteriorly-added ctenidia, *i.e.,* no trace of numerical asymmetry involving the posterior gills was detected—either in *Chaetopleura apiculata* or *Lepidochitona cinereus.*

A further pallial variation was noted in *Chaetopleura.* Although critical measurements were not made, a subjective but positive impression was gained that within the populations of *Chaetopleura* studied, two distinct forms occur—differing in the extent to which the pallial grooves are occupied by ctenidia. This was irrespective of total ctenidial number and size. In one type, the bases of the gills extend forward to the level of the head fold. This would conform to the accepted diagnosis of the genus *Chaetopleura.* In the other form, ctenidial bases extend forward for only two-thirds of the length of the pallial grooves (*i.e.,* to the level of the anterior quarter of the foot). Thus in this form, the anterior third of the pallial groove on each side is a naked gutter, and the whole pallial arrangement approximates that pertaining in chitons like *Lepidopleurus.* In the sample on which Figure 2A is based, 18 out of 76 specimens of *Chaetopleura* were asymmetric in ctenidial numbers. An earlier count of *Chaetopleura* from the same locality, which cannot be shown in the above figure since weights were not determined, gave three asymmetric out of 32. This gives a grand total for *Chaetopleura* of 21 asymmetric out of 108, or 19.5%.

Lepidochitona cinereus was chosen among British chitons, both because a preliminary survey showed a high incidence of asymmetry in the species, and because a population was already being sampled for gonadial studies. Figure 2B shows that only 65 out of a sample of 126 chitons were symmetrical, and that the asymmetries in one case involved a difference of three gills (15:18), in nine cases involved a difference of two gills, and in 51 cases a difference of one gill. Actual asymmetries are commonly more extensive than the figures for *Lepidochitona cinereus* indicate. Thus, often an 18:18, or 17:17, or 16:16 count includes one relatively large anterior (last-formed) gill on one side, and a minute (though differentiated) anterior gill on the other. In *Lepidochitona* as in *Chaetopleura,* there is clearly a correlation between adult size, expressed as wet tissue weight, and

FIGURE 2. Number of ctenidia on each side of the body in relation to "wet tissue weight" in chitons (weights in milligrams on the ordinate are plotted logarithmically); closed circles (●) indicate chitons which are symmetrical (*i.e.,* which have the same number of ctenidia on left and right sides); horizontal lines indicate asymmetrical chitons and link open circles (○) at the appropriate gill numbers. 2A, for *Chaetopleura apiculata* from Woods Hole, Mass.; 2B, for *Lepidochitona cinereus* from the Firth of Forth, Scotland.

ctenidial number. For *L. cinereus* in this particular population, there is rather better circumstantial evidence relating size to age. When the weight distribution of Figure 1B is plotted on probability paper (as used by Harding, 1949, and applied by Hunter, 1961, to snail populations) it shows a polymodal distribution with four significant peaks. However, length distributions for several other samples from this population were prepared in connection with studies on fecundity and, when these are plotted, show a clear trimodality which can be attributed to a 1+ (*i.e.*, more than one year old, *e.g.*, in a September sample such as the one used for weights and ctenidial counts—about 14–15 months old), a 2+ and a 3+ age group. It is notable that the youngest (1+) specimens of *Lepidochitona* already have from 11 to 15 gills on each side. No immediately post-larval material of *Lepidochitona* was available. In summary, numerical asymmetry in *Lepidochitona* involved 61 out of a sample of 126 individuals or 48.4% of the population, and qualitative asymmetry involved a still greater proportion of the population.

Certain general observations can be made. Except for a few very small specimens of each species, most of the *Chaetopleura* and *Lepidochitona* examined were adult. Growth continues in adult chitons, and apparently the development of additional ctenidia anteriorly also continues. There is a real correlation between gill number and adult size. However, in these samples at least, there is no indication of any increase in asymmetry with increasing size. The relation of left to right sides in both species is summarized in Table I.

TABLE I

Extent of ctenidial asymmetry in two species of chitons

	Nos. of individuals							
	With extra left gills			Symmetric	With extra right gills			Totals
	3	2	1		1	2	3	
Chaetopleura apiculata	0	2	8	87	9	2	0	108
Lepidochitona cinereus	0	5	24	65	27	4	1	126

In the course of gill-counting in *Chaetopleura* and in *Lepidochitona*, the few specimens which showed damage to a ctenidial row, or repair thereof, were rejected. In the 234 specimens of the main series, three cases were encountered in which ctenidial axes were split. In one specimen of *Chaetopleura*, the last (posterior) gill on the left side was biaxial, and in another, the third and fourth gills from the posterior end of the right row were both triaxial. In one specimen of *Lepidochitona* the last gill on the right side was triaxial.

Certain other chitons were examined in less detail. These included small samples of four other species of chitons from Scottish waters, which were examined before the main series of *Lepidochitona cinereus* was prepared. Eleven specimens of *Lepidopleurus cancellatus*, dredged from 18 m. off Dun Chonnuill in the Garvelloch Islands (see Hunter and Muir, 1954), had lengths ranging from 3.3 to 7.6 mm., had gill numbers from 8 to 11 per side, and included three asymmetric specimens (11L:10R, 11L:10R, and 9L:10R). A fresh sample of the commoner Clyde species of this genus (*L. asellus*) was not available but six specimens in a museum collection proved to be all symmetrical with from 10 to 13 gills on each side. A sample of nine specimens of *Tonicella marmorea* from near Shandon, on the eastern shore of Gareloch, ranged in size from 12.5 to 23.7 mm., had gill numbers from 17 to 28 per side, and included one asymmetric individual (23L:24R). Samples of *Lepidochitona cinereus* from this last locality, and from two localities in the Firth of Forth showed that nearly half of each population was asymmetric in gill numbers. Since *L. cinereus* is also the most abundant intertidal Scottish chiton, it was decided to use this species along with *Chaetopleura apiculata* in the main series. Lastly, 15 specimens of *Acanthochitona crinitus* (formerly known as *Acanthochitona fascicularis*), collected from near low water mark on a boulder beach just south of Port Appin, Argyllshire, ranged in length from 6.1 to 13.0 mm., had gill numbers from 14 to 17 per side, and included two asymmetric individuals (both 15L:16R). Thus, of five species of Scottish chitons examined, four proved asymmetric in ctenidial numbers. It is worth noting that these five species include representatives of both the major orders of living Polyplacophora. Three genera: *Lepidochitona* and *Tonicella* (both in family Lepidochitonidae), and *Acanthochitona* (family Cryptoplacidae) are placed in the Order Chitonida, the group containing the more typical "modern" chitons usually living on rocky surfaces in the intertidal zone. The genus *Lepidopleurus* (family Lepidopleuridae) is placed in the Order Lepidopleurida, which includes some of the most ancient of polyplacophoran genera (*e.g., Helminthochiton,* from Lower Ordovician) as well as four surviving genera which live offshore and in deeper waters (extending to beyond 6000 m.).

Subsequently, commercially obtained samples totalling 65 specimens of a moderately large chiton, *Katherina tunicata* from the Pacific Coast, probably collected near Gladstone, Oregon, were examined. Of these, 24 specimens proved to have ctenidial or pallial damage, and weights and gill counts were determined on the remaining 41. Wet weights ranged from 13.8 to 55.8 g., and gill numbers from 46 to 61 per side. Only 22 out of 41 specimens of *Katherina* were symmetrical, and the asymmetries (46.3% of the population) in two cases involved a difference of three gills, in five cases a difference of two gills, and in 12 cases a difference of one gill.

Few previous authors have studied ctenidial numbers in chitons. In a footnote, Pelseneer (1897) refers to his finding single specimens of six species of Atlantic chitons, each with an asymmetry of one gill. His species were *Acanthochitona discrepans, A. zelandicus, Lepidopleurus cajetanus, L. articus, "Boreochiton marmoreus"* (=*Tonicella marmorea,* above), and *Ploxiphora coelata.* Perhaps the only previous detailed examination of a population was by Snyder and Crozier (1922) on *Chiton tuberculatus* at Bermuda. These authors examined 100 specimens, and in individuals ranging in size from one to ten cm. long, found ctenidial numbers from 32 to 54 on each side with 69% of the sample asymmetrical.

Thus, in four species of chitons studied in detail, the percentages of asymmetrical specimens found have been 19.5% (*Chaetopleura apiculata*), 46.3% (*Katherina tunicata*), 48.4% (*Lepidochitona cinereus*), and 69% (*Chiton tuberculatus*). In addition, asymmetries of ctenidial numbers occur in at least eight other species.

DISCUSSION

As noted in the introduction, the discovery of *Neopilina* has reopened discussion of possible metamerism in ancestral molluscs. Chitons show replication in several organ systems—including shell valves, pedal musculature, nervous system, circulatory system, and ctenidia. As a result of their anatomical studies of *Neopilina,* Lemche and Wingstrand (1959b; see also Lemche, 1957, 1959b) proposed that such features of chitons, as well as similar structures in *Neopilina,* were primitive metameric characters. Lemche (1959a, 1959c) has gone so far as to detail homologies between a primitive arthropod triramous appendage and the gill in *Neopilina,* and between this latter and the ctenidia in the rest of the Mollusca (Lemche, 1959b).

Of course, the hypothesis that the molluscan ancestor was a short segmented animal has been advanced before (see, *e.g.,* Pelseneer, 1899, 1906; Naef, 1926). These older protagonists of a segmented ancestor drew their evidence largely from morphological studies of the coelomic derivatives, notably reno-pericardial and genital ducts, in chitons and cephalopods. The significance of *Neopilina* in relation to such theories was stressed by Lemche and Wingstrand (1959a, 1959b). They suggested that the ancestral mollusc must have shown relatively complete metamerism, that this is present to a somewhat reduced extent in *Neopilina,* that this is still further reduced in chitons, and that this metamerism degenerates so completely as to be undetectable in the rest of the molluscs.

For about 30 years the original metameric hypothesis, set up by Naef and Pelseneer among others, was abandoned. This resulted from the extensive and convincing work of the molluscan comparative morphologists, such as Yonge, Graham, and Fretter, whose studies of ciliary mechanisms, ctenidial blood-vessels, reno-pericardial and genital ducts in the more primitive gastropods (as summarized in Yonge, 1947) indicated a very different pattern of ancestral mollusc. Although primitively bilaterally symmetrical, this animal was totally unsegmented and possessed a posterior mantle-cavity enclosing a pallial complex of paired structures which included two ctenidia. The setting up of such a hypothetical ancestor had, of course, required consideration of those cases of molluscs in which there are more than two ctenidia, *i.e.,* the primitive cephalopod *Nautilus* (see Yonge, 1947) and all the chitons (Yonge, 1939). Such consideration led to the conclusion that these cases represent *secondary* replications of structures of the ancestral mollusc. The publication of the beautifully detailed anatomical account of *Neopilina galathea* (Lemche and Wingstrand, 1959b), has made review of concepts of molluscan evolution inevitable. Few students of the Mollusca would accept the more extreme homologies proposed by Lemche (1959a, 1959b, 1959c), which equate structures in the more primitive molluscs with corresponding annelid parts. Fretter and Graham (1962), in their excellent and detailed synthesis of existing knowledge of functional morphology in the prosobranch gastropods, have accepted the concept that *Neopilina* shows metamerism and have linked this to the older theory of a segmental ancestor. They also support the concept that the mantle-cavity was most primitively a groove bounded by the mantle edge and surrounding the head-foot, rather than a posterior cavity. Yonge (see Morton and Yonge, 1964) has denied that metamerism occurs in *Neopilina* and, although allowing that the ancestral mantle-cavity might have been a pallial groove, adheres essentially to the concept of the ancestral mollusc outlined above. Obviously, aspects of these theories are mutually exclusive. The pattern of molluscan evolution, involving successive reductions of metameric structures, proposed by Lemche and Wingstrand (1959a, 1959b) requires as a premise that the multiplied organ-systems of chitons represent a simplification of more extensive metamerism.

Therefore, the question must be asked: do chitons show any vestiges of true metamerism? A seemingly negative answer is provided by existing evidence on the development of shell plates, on variation in auriculoventricular connections in the Class, and by the data on ctenidial numbers presented in this paper.

As regards shell plates, their development in several chitons is similar. The elongate trochophore develops a mantle rudiment, which about the time of settlement secretes six shell plates. After an interval, a larger plate is added *anterior* to the series. Still later, a small last plate is added at the *posterior* end of the series. At no stage in the development is there a "budding zone" for shell plates. This sequence of events, known for several chitons, has recently been confirmed for *Chaetopleura apiculata* by one on the present authors (S. C. B.).

As a Class, the chitons show significant variation in the afferent chambers of the heart. These variations were surveyed by Pelseneer (1897, 1906). The pericardium is always dorsal and posterior, and contains an elongate ventricle in the midline which receives blood from two symmetrical elongate auricles. In a minority of chiton genera, Pelseneer found that there are single auriculoventricular (A-V) openings on either side; in the majority of genera there are two A-V openings on either side; while in *Chiton squamosus* there are three pairs and in *C. goodalli* four. Pelseneer also remarks that numerical asymmetries of the A-V openings can occur, although they are apparently extremely rare. It would be difficult to relate the paired auriculoventricular openings to any external "segmentation" such as shell valves. Similarly, although the renal organs of chitons are lobulated, relating particular lobes to external "segments" or to the renal lobes and discrete "nephridiopores" of *Neopilina* would be equally difficult.

Turning to the ctenidia of chitons, their numbers, though approximately fixed for each species, show variations which are germane to the question of metamerism. First, gills are added anteriorly and somewhat irregularly as growth proceeds. Second, there can be marked asymmetry between left and right ctenidial rows in individual specimens (from 19.5% to 69% in four species studied in detail). Third, actual asymmetry of development can be more marked than mere enumeration of ctenidia would indicate, since the degree of differentiation of the anteriormost gills often varies. Fourth, the "definitive" ctenidial number in different species of chitons ranges from 8 to 160. (No one has ever suggested a primitive mollusc with 80 segments.) Finally, individual ctenidia cannot be related to any other replicated structure, still less allocated to specific segments.

The broad phyletic significance of the above can now be summarized. If no true metamerism occurs in primitive molluscs, the closest connections and possible origins of the phylum lie in the turbellarian-rhynchocoele phyla (as is concluded by Yonge, 1947; Morton and Yonge, 1964). If metamerism was a feature of the primitive mollusc, connections should be sought with the annelid-arthropod phyla (as urged by Lemche and Wingstrand, and partially accepted by Fretter and Graham, 1962). No one has attempted to establish a connection between molluscs and either of the other metazoan groups showing segmentation: the Cestoda or the Chordata. At this point, it is appropriate to try to define metameric segmentation as it is found in the Annelida and Arthropoda. Hyman (1951, pp. 28–29) offers several cogent phrases in defining segmentation as "a serial succession of sections, each of which contains identical or similar representatives of all the organ systems of the body" in which "all parts . . . are serially repeated at regular intervals" and "the segments form in an anteroposterior sequence so that the first segment is the oldest, the last the youngest." Thus, the essence of metamerism is the serial succession of segments, each containing unit-subdivisions of the several organ systems.

The most detailed homologies between chitons and *Neopilina* proposed by Lemche and Wingstrand (1959) involved the alleged metamerism of the gills and circulatory organs of both (although clearer "segmentation" in *Neopilina* is found in the coelomic cavities and their genito-renal derivatives). A major implication of the results presented above is that "segmentation" in chitons is more apparent than real. It is difficult to imagine morphogenesis of any metameric sort which would allow the addition *anteriorly* of the penultimate shell valve to the six already developed, and this followed finally by the laying down of the last (eighth) valve *posteriorly*. Similarly it is difficult to invoke metamerism with regard to the anterior addition, irregularly and independently on either side, of ctenidia as growth proceeds. Finally, it is difficult to characterize as a metamerically segmented animal: a chiton with 8 shell plates, with two, three or four auriculoventricular openings, with 21 ctenidia on one side and 23 on the other (or 15 and 18), and with a ladder-like nervous system of irregular transverse connectives—all these arranged apparently independently of each other. There is little of the serial succession of segments, each containing unit-subdivisions of the several organ systems, about such replications as are found in chitons. Thus, it seems most probable that the multiplied structures of chitons reflect functional replication rather than ancestral segmentation.

Even if we can thus dismiss true metamerism as regards chitons, the question of "segmentation," both in *Neopilina* and in a hypothetical molluscan ancestor, remains. Similar arguments to those presented above can be used to criticize the concept of metamerism applied to the described structures of *Neopilina*. *Neopilina* has five pairs of gills, two pairs of auricles, six pairs of nephridia, one or possibly two pairs of gonads, 8 pairs of pedal retractor muscles, 10 sets of lateropedal nerve connections, and a single shell with a coiled protoconch. As Yonge has pointed out (Yonge, 1957, 1960; Morton and Yonge, 1964), final assessment of the phyletic position of *Neopilina* must await embryological studies—particularly on the development of the allegedly metameric structures, and functional studies on the organization of the gills in living *Neopilina*, which could establish whether or not these structures are homologous with the ctenidia of the other molluscs.

The question of the ancestral stock can be re-examined. According to the classical picture, derived from studies on the functional morphology of the most primitive living gastropods (Yonge, 1947), the ancestral mollusc had a posterior mantle-cavity containing one pair of ctenidia connected to one pair of auricles. However, even before the discovery of *Neopilina*, there were several difficulties in relating this model to conditions in the primitive cephalopod *Nautilus*—with two pairs of ctenidia and four auricles, and in the chitons—with many ctenidia and two elongate auricles (with two, four, or six auriculoventricular openings). There is a striking resemblance in hearts (each with four auricles and a "single" ventricle) between *Neopilina* and *Nautilus*. There is a further, though less obvious, resemblance of both to conditions as they exist in the majority of chitons—with four auriculoventricular connections. Perhaps surprisingly, the arrangement of heart chambers and of their interconnections seems to form a relatively conservative feature in the evolution of other molluscan groups. For example, within the Order Neritacea, evolution of fresh-water and terrestrial forms has occurred completely independently of—though occasionally parallel to—the rest of the gastropods (Morton and Yonge, 1964; Hunter, 1964). The course of this evolution has involved the usual gastropod reduction of symmetrically paired structures. However, comparative studies on certain Neritacea by one of the present authors (W. R. H., unpublished) have shown that even in many forms where this reduction is complete for almost all of the other structures, a small right auricle remains, in addition to the functionally important left one.

In a footnote in the classic paper of Yonge (1947), Dr. C. F. A. Pantin inquired if it was necessary to postulate the possession of only a single pair of ctenidia in the primitive mollusc. In view of the heart morphology of *Neopilina, Nautilus* and chitons discussed above, it would seem as justifiable to set up a model ancestral mollusc with four ctenidia and therefore four auricles. Subjectively, a four-fold basic organization would seem a more reasonable starting point for two sorts of morphogenesis: involving either reduction to one pair or replication to many. Both a line of organisms with one gill on either side, and a line with many could thus evolve from an ancestral stock with two gills on either side.

While it is realized that the setting up of yet another hypothesis, with four as the ctenidial number in the stem-mollusc, is but tenuously based on the observed phenomena of replication of parts in chitons, it is emphasized that the conclusion that chitons are *not* metameric is much more firmly established.

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SUMMARY

1. The discovery and description of *Neopilina* has reopened discussion of possible metameric segmentation in the primitive molluscs. This has involved reconsideration of the multiplied organ systems in chitons, which some authorities regard as metameric in origin. The length-weight relationship was determined and ctenidial numbers counted for populations of two species of chitons: *Chaetopleura apiculata* from Cape Cod, and *Lepidochitona cinereus* from the North Sea coast of Scotland. Less detailed observations were made on other species of chitons and on other organ systems. Growth continues in adult chitons, together with the addition of ctenidia anteriorly, so that there is a correlation between gill-number and adult size. *Chaetopleura apiculata* adults, weighing from 38 to 1324 mg., had

gill numbers ranging from 17 to 25 per side. *Lepidochitona cinereus* adults, weighing from 6 to 340 mg., had gill numbers ranging from 11 to 20 per side. Asymmetry in ctenidial numbers between the left and right sides of single specimens occurred in 19.5% of *Chaetopleura apiculata*, and in 48.4% of *Lepidochitona cinereus*. Qualitative asymmetry was even more extensive in the populations studied. Since such marked ctenidial asymmetries as reported above occur, and since individual gills are added independently on either side, the ctenidia of chitons *cannot* be paired structures. The organization of certain other replicated structures (including shell-plates, auriculoventricular connections, and renal lobes) is considered more briefly, drawing on earlier pertinent work, and leads to the obvious result that individual ctenidia cannot be related to any other replicated structure—still less allocated to specific "segments."

2. These findings are discussed in relation to the alleged metamerism of chitons, and it is also concluded that such replicated organs *cannot* represent a simplification of a more extensive metamerism. Further, it is shown that it is possible to criticize the concept of metamerism as applied to the described structures of *Neopilina*. The phyletic significance of this is explored, and it is concluded that evidences for a connection between the primitive molluscs and the turbellarian-rhynchocoele phyla are better than are those for metamerism and an annelid-arthropod connection. A model ancestral mollusc with a *four-fold* basic organization (*e.g.*, four ctenidia, four auricles, four renal organs, etc.) is proposed. It is stressed that, while *any* model of an ancestral mollusc is highly speculative, the evidences against metamerism in chitons (and probably in all primitive molluscs) are overwhelmingly strong.

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